IDENTIFICATION OF NOVEL PROGNOSTIC BIOMARKERS IN DIFFUSE LARGE B CELL LYMPHOMA IN THE RITUXIMAB ERA

by

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Abstract

Diffuse large B cell lymphoma (DLBCL) is the most common form of lymphoma and is characterized by marked clinical and genetic heterogeneity. Approximately 60% of patients with DLBCL are cured with multi-agent chemotherapy consisting of rituximab, cyclophosphamide, hydroxyldaunomycin, oncovin and prednisone (R-CHOP). R-CHOP represents the current standard of care throughout the world for the treatment of DLBCL. The international prognostic index (IPI) is a clinical tool that can help risk-stratify patients at the time of diagnosis but it fails to identify 50% of patients who will relapse and provides no insights into the biology of the disease. The aim of this work was to identify prognostic markers that would complement the IPI, would reflect the underlying tumour biology and could be easily translated into the clinical setting. We used flow cytometry (FCM) to study the protein expression of the CD20 antigen, the target of rituximab on lymphoma cells and performed fluorescence *in situ* hybridization on DLBCL biopsies to identify the presence of genomic rearrangements in the *BCL2* and *MYC* oncogenes. We also used DNA sequencing to determine if somatic mutations involving the rituximab binding epitope of CD20 had any impact upon patients failing therapy. We determined that recurrent mutations of exon 5 of CD20 do not explain rituximab resistance in clinical cases of DLBCL. In contrast, we could demonstrate that both a reduced expression of CD20 by FCM and the presence of concurrent de-regulation of *MYC* and *BCL2* expression are independently associated with an inferior survival in DLBCL patients treated with R-CHOP. Importantly, both maintain their

prognostic significance in multivariate analysis, independent of the IPI. Furthermore, these biomarkers reveal important novel insights into DLBCL biology and provide rational targets for therapy. As such they should be investigated and validated prospectively for future use in the clinical setting.

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Abbreviations

Abbreviation	Definition
ABC	activated B cell subtype of DLBCL
ADCC	antibody-dependent cellular cytotoxicity
AID	activation induced cytidine deaminase
BCCA	British Columbia Cancer Agency
BCLU	B cell lymphoma, unclassifiable, with features intermediate between BL and DLBCL
BCR	B cell receptor
BL	Burkitt lymphoma
BM	bone marrow
CDC	complement-dependent cytotoxicity
CHOP	cyclophosphamide, hydroxyldaunomycin, oncovin and prednisone
COO	cell of origin
CSR	class switch recombination
CT	computed tomograms
D	Diversity
DCD	direct cell death
DLBCL	diffuse large B cell lymphoma
ECOG	Eastern Cooperative Oncology Group
FCM	flow cytometry
FFPET	formalin fixed paraffin embedded tissue
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
FL	follicular lymphoma
GC	germinal center
GCB	germinal centre B cell
GEP	gene expression profiling
IG	immunoglobulin gene
IHC	immunohistochemistry
IPI	international prognostic index
ISCN	International System for Human Cytogenetic Nomenclature

J	Joining
LDH	lactate dehydrogenase
MZL	marginal zone lymphoma
NHL	non-Hodgkin's lymphoma
Non-GCB	Non-GCB subtype of DLBCL
OS	overall survival
PB	peripheral blood
PCD	programmed cell death
PE	phycoerythrin
PFS	progression free survival
PMBCL	primary mediastinal large B cell lymphoma
R	rituximab
RAG	recombination activating genes
R-CHOP	CHOP and rituximab
RMA	robust multi-chip analysis
SHM	somatic hypermutation
SLL	small lymphocytic lymphoma
SNP	single nucleotide polymorphisms
TMA	tissue micro-array
V	Variable
WHO	world health organization
WTSS	whole transcriptome shotgun sequencing

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Dedication

To Greg, Leonie and Justin for their love, patience, and support.

Co-authorship statement

I designed and performed the research, analyzed the data and wrote all of the manuscripts included in the thesis. I selected the lymphoma cases for each study based on the availability of tissue, extracted the DNA/RNA, performed the gene expression profiling and the sequencing experiments needed for these manuscripts. Co-authors contributed to the studies by extracting DNA/RNA from frozen tissue, performing gene expression profiling, FISH assays and the bioinformatics analyses required for these manuscripts. Clinical colleagues provided specimens, reviewed the diagnoses and gathered clinical data. The primary investigators help design the research and all authors reviewed and contributed to the manuscripts.

Chapter 1: Introduction

1.1 Diffuse large B cell lymphoma

1.1.1 Epidemiology

Lymphoma is a cancer that originates from the lymphocytes within the immune system. It usually arises in lymph nodes and other lymphoid organs such as the spleen and bone marrow but can occur at any other site within the body. Lymphomas represent the fifth most common type of cancer in British Columbia (http://www.bccancer.bc.ca/HPI/CancerStatistics/FF/default.htm). Diffuse large B cell lymphoma (DLBCL) is the most common lymphoma subtype, representing approximately 30% of cases and its incidence is rising[1]. DLBCL can occur after histological transformation of a previous indolent lymphoma such as follicular lymphoma (FL), marginal zone lymphoma (MZL) or small lymphocytic lymphoma (SLL). However, the current work focuses on the more common "*de novo*" DLBCL, not otherwise specified, according to the revised World Health Organization (WHO) criteria of 2008[1].

1.1.2 Diagnosis

The accurate histological diagnosis of lymphoma is crucial because it determines the choice of therapy and thus is best made by an experienced hematopathologist [1,2]. The diagnosis is based mainly on routine morphologic evaluation and is often complemented by immunohistochemistry (IHC). The detection of chromosomal alterations by G-banding or fluorescence *in situ*

hybridization (FISH) can sometimes be useful in distinguishing lymphoma subtypes. Many centers also use flow cytometry (FCM) to immunophenotype lymphoma cells, a technique that can identify antigens expressed on the outer cellular membrane and which can further help to characterize the lymphoma subtype.

1.1.3 Clinical evaluation and prognostic factors

Once the diagnosis of DLBCL is established, the patient is staged according to the Ann Arbor classification[3]. This is accomplished by performing a bone marrow biopsy and imaging studies that usually includes computed tomograms (CT) of the chest, abdomen and pelvis to determine the extent of tumour involvement. Stages < II represent disease confined to one side of the diaphragm whereas stages III and IV represent more wide spread disease +/- dissemination to other organs (IV). The tumour stage also depends on the absence of constitutional "B symptoms" which includes at least one of the following: > 10% unexplained weight loss within the previous 6 months, fevers > 38°C and night sweats. Proper staging is not only an important clinical prognostic factor but is used to determine the total number of cycles of chemotherapy (+/- radiation therapy) and to evaluate the clinical response to therapy.

The international prognostic index (IPI) is the most powerful tool currently used in clinical practice to predict a patient's response to therapy at the time of diagnosis[4]. This tool predicts the probability of survival based on the presence of

5 factors where one point is assigned for each of the following: age > 60 years old, stage > II, serum lactate dehydrogenase (LDH) > normal range, patient's performance status > 1 based on the Eastern Cooperative Oncology Group (ECOG) criteria and greater than one extra-nodal site. Low-risk patients (IPI = 0-1) have 5 year overall survival (OS) rates in the order of ~75% while high-risk patients (IPI =4-5) have 5 year OS of ~ 25%. Although newer therapies that include rituximab have modified survival risks, the IPI still remains the gold standard for prognostication[5]. Therefore, to be considered clinically useful, novel biological markers must demonstrate that they have prognostic value independent of the IPI in multivariate analysis.

1.1.4 Therapy

In the 1970s, multi-agent chemotherapy regimens were tested and found to cure a subset of patients with DLBCL[6,7]. This led to the adoption of the regimen containing cyclophosphamide, hydroxyldaunomycin, oncovin and prednisone (CHOP)[8]. A number of so-called 3rd generation regimens were developed in the 1980s and were considered to be superior to CHOP, but a phase III randomized clinical trial comparing several of these to CHOP failed to show superiority in OS. Importantly, CHOP was less toxic than more intensive regimens and therefore remained the standard of care for almost 30 years[9]. A paradigm shift occurred in 2001 when rituximab, an antibody directed toward the CD20 antigen on B lymphocytes, was added to CHOP. Rituximab's main mechanism of action is by eliciting an immune response which triggers tumour cell death by cell dependent

cytotoxicity and complement dependent cytotoxicity although direct apoptosis can also occur (reviewed by Bonavida)[10]. The new R-CHOP regimen was found to be associated with a significant improvement in both OS and progression free survival (PFS) in patients with DLBCL in a number of randomized phase III clinical trials[11]. A population-based study in British Columbia demonstrated that the introduction of R-CHOP in 2001 improved the survival of patients with DLBCL by more than 50% (5 year OS in pre-rituximab era ~ 40% compared to post-rituximab era ~ >60%, see Figure 1.1)[12]. The number of cycles of R-CHOP therapy administered to patients typically depends on stage and response to therapy. Patients with limited disease may be treated with an abbreviated course of chemotherapy (e.g. 4 cycles of R-CHOP) followed by involved field radiation, while patients with disseminated disease are treated with 6-8 cycles of R-CHOP[13].

1.1.5 Lymphoma relapse

Although rituximab has made a tremendous impact on the lives of patients with DLBCL, there are patients that fail to achieve a complete remission following R-CHOP induction therapy. These patients have primary refractory disease and have a very poor prognosis because they usually don't respond to subsequent cytotoxic agents[14]. There are additional patients whose lymphoma relapses after having achieved a complete remission. Relapses usually occur within the first 2-3 years after diagnosis. Although a subset of patients can be cured with a hematopoietic stem cell transplant at the time of relapse, the majority of patients

have poor clinical outcomes regardless of the salvage chemotherapy regimen chosen[14,15]. Therefore, unlike other lymphoma subtypes, patients with DLBCL are given frontline therapy that provides the best chance of cure at the time of diagnosis. In 2010, the standard treatment for DLBCL in North America is R-CHOP.

1.2 Normal B cell biology

DLBCL cells are derived from the germinal centre (GC) and as such retain many of the normal GC B cell characteristics. These include increased proliferative capacity, resistance to apoptosis, ongoing somatic hypermutation and a requirement for interaction with cells within the lymph node "microenvironment". To fully grasp DLBCL biology, an appreciation of the normal germinal centre processes is required.

1.2.1 B cell development

B lymphocytes evolve from a common lymphoid progenitor cell in the bone marrow. Here they undergo rearrangement of their immunoglobulin (*IG*) genes, which are composed of a number of discontinuous variable (V), diversity (D), joining (J) and constant segments. The process of VDJ rearrangement is the main mechanism for generating functional and unique B cell receptors (BCR) that have different affinities to various antigens[16]. The double stranded DNA breaks required in this process are created by the enzyme recombination activating

genes 1 and 2 (RAG-1/2)[17,18]. A functional BCR is required for B cell differentiation and survival once a B cell leaves the bone marrow[19].

1.2.2 B cell differentiation and surface receptors

As B cells develop and differentiate into mature antibody producing plasma cells, they express different cell surface receptors. There are approximately ten B cell-specific molecules that have been named according to an internationally recognized nomenclature designated by "clusters of differentiation" or CD[20]. Herein, I will briefly describe the most relevant proteins for this thesis. CD20 was the first B cell antigen to be discovered (initially termed B1) and is the target of rituximab[21]. CD20 is present on the surface of mature B cells and its functions are not fully elucidated although there is evidence that it may act as a calcium channel. The BCR complex consists of two non-covalently bound receptors CD79a (Igα) and CD79b (Igβ) that contain tyrosine phosphorylation residues and Src family kinase sites that are essential for BCR signalling [20,22,23]. CD19 is a pan-B cell marker that functions to amplify and regulate the Src-family kinase activity at the BCR. CD5 is an antigen typically found on the surface of T cells; however, a minor circulating mature B cell population can also co-express CD19 and CD5. These cells were initially called B-1a cells, are part of the innate immune system and participate as a first line of defence against bacterial infections. DLBCL cells express mature B cell receptor antigens, CD19, CD20, CD79a and CD79b. Co-expression of CD5 with CD19 is typically associated with CLL and mantle cell lymphoma, but can also be seen in 8-10% of DLBCL.

1.2.3 The germinal center reaction

In response to T cell dependent antigens, B cells migrate into GCs to undergo a variety of processes affecting *IG* genes with the goal of producing high-affinity antibodies to this newly encountered antigen (see Figure 1.2). Here the B cells undergo rapid clonal expansion, a process mediated by cytokines provided by cells within the GC microenvironment such as T cell and antigen presenting cells (dendritic cells) (reviewed by LeBien & Tedder and Klein *et al*.)[20,24]. The rapidly proliferating large B cells, called centroblasts, diversify their *IG* genes by somatic hypermutation (SHM) and class switch recombination (CSR), both processes mediated by the enzyme "activation-induced cytidine deaminase" (AID)[25]. AID transforms a CG pair to a UG pair with the subsequent low-fidelity mismatch repair mechanism leading to a range of mutations or even double-strand breakage that is required for CSR. This combination of rapid cellular proliferation and mutation enables the emergence of a multitude of B cells with BCRs showing varying affinity for the antigen. Cells producing the highest affinity antibody are destined to become plasma cells, which secrete large quantities of immunoglobulin, or memory B cells that respond rapidly upon repeat exposure to antigen. Cells producing low-affinity antibody or with crippling mutations in their *IG* genes are depleted by apoptosis[24]. This GC reaction is regulated by the coordinated expression of several transcription factors, including BCL6, PRDM1, IRF4, LMO2, E2A, XBP1 and PAX5.

1.2.4 The GC: A genotoxic environment in DLBCL

The GC is an environment favourable to DNA strand breaks, cellular proliferation and inhibition of apoptosis. To ensure a timely response to pathogens, centroblasts have the capacity for rapid proliferation with cell cycle times as short as six hours[26]. This is achieved by the up-regulation of genes involved with proliferation and down-regulation of genes involved with inhibition of the cell cycle[27,28]. Additionally the replicative ability of cells within the GC is maintained by the expression of telomerase, an enzyme responsible for the maintenance of the telomeres that are necessary for successful cell division [29]. The presence of genomic damage induced by SHM or CSR would normally elicit a DNA damage response leading to either apoptosis or cell cycle arrest allowing for DNA repair however, these processes are suppressed in GC B cells by decreasing the expression of P53 and BCL2 thus enabling the GC reaction to proceed unimpeded[24,30]. Furthermore, AID is not specific for the *IG* genes and has been shown to generate DNA strand breaks or mutate other genes[31]. Indeed, AID is required for the generation of the chromosomal translocations affecting *BCL2, BCL6* and *MYC* that are commonly detected in DLBCL.

1.3 DLBCL pathophysiology

1.3.1 DLBCL molecular subtypes

Despite having similar morphologic characteristics, DLBCL represents a biologically diverse group of tumours. Over the past decade, our understanding of DLBCL biology has improved such that this disease can be classified into at

least 3 molecular subtypes based on similar gene expression patterns. These cell of origin (COO) distinctions identify at least three DLBCL subtypes that correspond to distinct stages of lymphocyte differentiation including: 1) germinal centre B cell (GCB); 2) activated B cell (ABC), and 3) primary mediastinal large B cell lymphoma (PMBCL)[32,33]. The GCB subtype has a favourable clinical outcome and demonstrates a gene expression profiling (GEP) pattern that mimics normal GC B cells (i.e. high expression of *BCL6, GCET, HGAL* and *CD10*). The ABC subtype has a less favourable clinical outcome and displays a GEP that mimics a cell just prior to GC exit, with a signature containing genes characteristic of activated B cells (high expression of *MUM1, FOXP1* and *BLIMP1*) and some plasma cell genes. The ABC subtype also exhibit constitutive activation of NF-κB pathway genes[32]. The gold standard for classifying DLBCL cases according to cell of origin is using a Bayesian classifier to estimate the probability of a case being ABC versus GCB (see Figure 1.3)[34]. The expression patterns of some of these genes have been used to create algorithms capable of assigning molecular subtypes using IHC. The first IHC classifier algorithm proposed by Hans *et al*. used expression of BCL6, MUM1 and CD10 but this resulted in a 20% discrepant call rate between IHC and the "gold standard" GEP[35]. A new algorithm by Choi *et al.* uses more genes (GCET1, CD10, MUM1 and FOXP1) and has a higher concordance rate with GEP (93%)[36].

The PMBCL subtype is characterized by low expression of components involved in BCR signalling, and a profile resembling that of Reed-Sternberg cells found in classical Hodgkin lymphoma[33]. PMBCL is not the primary focus of this thesis.

An alternate gene expression model categorizes DLBCL into oxidative phosphorylation (Ox Phos), B-cell receptor/proliferation (BCR), and host response (HR) signatures[37]. This bioinformatics approach to molecular sub-classification lacks clinical correlation, has been relatively under studied and thus will not be used in the studies pertaining to this thesis.

GEP has been instrumental in identifying comprehensive patterns of expressed genes involved with cellular growth, differentiation and survival as well as interaction with the non-neoplastic elements in the tumour microenvironment. More recently, GEP has been performed in a large group of samples derived from patients treated with R-CHOP[38]. It not only confirmed that the COO based signatures remain prognostic in the rituximab era but demonstrated that new gene signatures, reflecting the composition of the stromal compartment, were also associated with clinical outcome, where the genes associated with the deposition of new blood vessels (stromal-2) was associated with an inferior prognosis[38]. In summary, studies using GEP have resulted in the discovery of a number of oncogenic signalling pathways that are important in DLBCL pathogenesis. Only the pathways relevant to the thesis will be discussed in detail here.

1.3.2 DLBCL oncogenic pathways

1.3.2.1 BCL2

BCL2 is a key inhibitory component of the intrinsic apoptosis pathway. In normal GC B cells, BCL2 expression is down-regulated to enable the apoptosis of cells with low-affinity BCRs, however BCL2 protein is abnormally expressed in >50% of DLBCL through a variety of mechanisms[39]. BCL2 translocations can occur in 20-30% of DLBCL cases and the t(14;18) translocation appear almost exclusively in the GCB type of DLBCL[40,41]. In contrast, amplification of the *BCL2* gene on chromosome 18q21 or de-regulation downstream of constitutive NF-κB pathway activation is more prevalent in the ABC type[42].

1.3.2.2 MYC

MYC is a master regulator of cell fate and its expression is normally tightly regulated because it has profound effects on gene transcription, especially as it relates to entry into the cell cycle. An excellent review by Meyer summarises the key discoveries in MYC research over the past 25 years and highlights the pervasive role of MYC in all cellular functions[43]. In addition to behaving as a transcription factor and regulating expression of genes important during G1 to S phase transition, MYC also exerts non-transcriptional control over DNA replication by interacting with the pre-replicative complex at sites of DNA synthesis[44,45]. *MYC* has also been shown to "re-program" the micro-RNA transcriptome by repressing the expression of most miRNAs with the exception of miR-17-92, which can increase the tumourigenic potential of lymphoma cells[46].

In DLBCL, *MYC* can be de-regulated through various mechanisms including translocation and gene amplification [40,47]. SHM-derived mutations in *MYC* are also common having been reported in approximately one third of DLBCL[31].

1.3.2.3 BCL6

The transcriptional repressor BCL6 orchestrates the GC reaction. As with *MYC* and *BCL2* it also is frequently de-regulated as a result of translocation in 30-40% of cases and mutated as a result of SHM[31,40]. BCL6 is normally expressed in the GC and therefore BCL6 protein expression is associated with a GCB DLBCL molecular subtype[35]. One of the main functions of BCL6 is attenuation of DNA damage response through transcriptional repression of *ATR*, *TP53*, *CHEK1*, and *CDKN1A* (p21), which permits the processes of SHM, and CSR to proceed without eliciting cell cycle arrest or apoptosis[48]. BCL6 also prevents terminal differentiation of GC B cells into plasma cells through repression of *PRDM1* expression [49]. Translocations and mutations prevent the normal process of BCL6 silencing at the termination of the GC response leading to a continuously proliferative, DNA-damage tolerant, maturation-arrested phenotype that sustains further genetic alterations induced by AID.

1.3.2.4 NFκB pathway

The NF-κB proteins constitute a family of inducible transcription factors that are major regulators of proliferation, differentiation, and survival of lymphoid cells (reviewed by Vallabhapurapu *et al.)*[50]. NF-κB members are normally maintained

in an inactive state by association with the cytoplasmic IκB family of inhibitory proteins. Physiologic activation of NF-κB occurs through either the canonical or alternate pathways, both of which induce phosphorylation of IκB leading to its ubiquination and proteolytic degradation in the proteasome. The degradation of IκB permits the translocation of NF-κB heterodimers into the nucleus where they can activate gene transcription of a large number of target genes. NF-κB induction through the canonical pathway normally occurs in response to infection or inflammatory cytokines acting through engagement of the antigen receptor (B cell receptor, T cell receptor or Toll like receptors) or following ligation of various cell surface receptors (IL-1R, CD40, CD30, TNFR1 and RANK), respectively.

The NF-κB pathway is constitutively activated in over 95% of ABC type and 47% of GCB DLBCL[51]. Several mechanisms contributing to NF-κB pathway activation have recently been identified. Mutations and deletions causing biallelic inactivation of *TNFAIP3/A20*, the negative regulator of NFκB, are described in approximately 25% of cases and attenuation of *TNFAIP3/A20* through promoter methylation in 40% of cases[51,52]. Activating mutations in *CARD11*, a key regulator of B cell receptor mediated NF-κB activation, have been described in approximately 10% of ABC DLBCL and 4% of GCB DLBCL[53]. Mutations in several other upstream regulators including *TRAF2*, *TRAF5*, *MAP3K7* and *TNFSF11A (RANK)* have also been reported to induce NF-κB activation[51]. Trisomy 3 or 3q amplification, containing *NFKBIZ*, (which enhances transactivation of some NF-κB target genes) and the *FOXP1* oncogene have

been reported in approximately one quarter of ABC DLBCL[54,55]. DLBCL may also up-regulate NF-κB through several other mechanisms including tonic BCR signalling, low-level constitutive engagement of CD40 and BAFF signaling[50,56-58].

1.3.2.5 B cell receptor signalling

Despite translocations and mutations involving *IG* loci, the majority of DLBCL cases retain functional BCRs and depend on constitutive activation of the BCR signalling pathway for tumour survival[59]. BCR signalling activates the SYK tyrosine kinase resulting in amplification of the original BCR signal and activates a number of downstream messengers including the SLP-65 adaptor molecule, and the Bruton Tyrosine Kinase (BTK). These effectors in turn lead to the activation of the Phosphoinositide 3-kinase (PI3K), PKC and the AKT pathways[60]. Recently, mutations in a critical residue in the functional ITAM motif of CD79B were detected in 18% of ABC type DLBCL biopsies[61]. These mutations were shown to increase BCR signalling in these tumour types by decreasing the feedback inhibition of Lyn kinase. The authors further demonstrated that Desatinib, a kinase inhibitor approved for the treatment of chronic myelogeneous leukemia, inhibits BCR signalling through the Src-family kinase and BTK and killed the ABC type cell lines with chronic active BCR signalling[61].

1.4 DLBCL prognostic factors in the R-CHOP era

1.4.1 Clinical utility of prognostic markers

There is a need to discover biomarkers that could reliably identify, at the time of diagnosis, DLBCL patients who will not respond favourably to R-CHOP as primary therapy. Although the IPI is useful to risk-stratify patients, there remains marked heterogeneity in clinical outcome within groups with equivalent IPI prognostic scores, especially in the high-risk group of patients. Risk stratifying patients based on a combination of clinical and biological markers could be useful in the context of clinical trials investigating more aggressive, Burkitt lymphoma-type regimens as primary therapy, or novel "targeted" therapies in the relapse setting[62]. Furthermore, high-risk patients' clinical responses to R-CHOP could be assessed after 1-2 cycles instead of at the end of cycle 4, thus allowing the physician to institute an early change in therapy and thereby avoiding the toxicity of additional cycles of R-CHOP.

1.4.2 Prognostic factors are therapy dependent: the rituximab effect

The addition of rituximab to CHOP has changed the prognostic significance of many of the biomarkers that were found to predict outcome in CHOP-only treated patients. The IPI has been re-evaluated in R-CHOP treated patients and although it maintains its prognostic significance, it fails to identify a significant proportion of patients who are considered high-risk (i.e. <50% chance of survival)[5]. A revised IPI (R-IPI) has been proposed which re-assigns IPI groups

3 to 5 to the high-risk category but the problem remains that these variables provide no insights into the underlying biology of the disease[5]. Rituximab was shown to provide a greater benefit to patients whose biopsies over-express BCL2 protein, such that BCL2 protein expression as a single biomarker, is no longer associated with a poor outcome[39,63]. Similarly, BCL6 protein expression, which was associated with a favourable outcome in the CHOP era, no longer predicts outcome in R-CHOP-treated patients[64]. Thus biomarkers are only useful when interpreted in the context of specific therapies, which can change dramatically over time.

1.4.3 Challenges of introducing prognostic factors in clinical practice

Currently, no prognostic biomarkers exist that could be used to complement the IPI. Many of the biomarkers evaluated thus far have been studied retrospectively and need to be validated by prospectively testing them in samples from an independent unbiased patient population, using uniform, robust and standardized methodologies. In addition, the technology must be universally available in clinical laboratories, with measures in place that ensure proper quality control before such tests can be introduced into the clinic, e.g. instrument calibration, establishing “normal” ranges, standards, etc. This is especially challenging because technology is evolving at such a rapid pace that the tools used to detect biomarkers may become obsolete before they reach the clinic. For instance, GEP using microarray technology has never made it into clinical practice for reasons listed above, but also because of sample requirement issues such as

the need for fresh-frozen tissue. However, the cost of novel sequencing technologies that can simultaneously measure gene expression, such as whole transcriptome shotgun sequencing (WTSS), may be dramatically reduced in the near future so as to render GEP by microarray technology obsolete[65]. Finally, even when a technique is routinely used, such as IHC, different staining techniques and interpretative scoring render some markers, like BCL6 protein expression, highly variable and poorly reproducible between different laboratories[66]. Thus, the classification of DLBCL by COO, although routinely used in the research setting, has never been clinically validated.

1.5 Thesis theme and objectives

There is a need, therefore, to identify biomarkers that could be rapidly introduced into the clinical setting and can predict outcome in DLBCL patients treated with R-CHOP. Furthermore, they should provide some insights into DLBCL biology beyond the IPI. Based on the strong evidence supporting cell surface signalling and oncogene activation in DLBCL pathophysiology, the objective of this work was to study the relationship between the expression of CD20, BCL2 and MYC with clinical outcome, using technologies that are currently available in the clinical laboratory including DNA sequencing, IHC, FCM and FISH.

1.6 Hypotheses

1.6.1 Hypothesis 1

Given the significant impact of rituximab on the outcome of patients with DLBCL, impairment due to a dysfunctional or absent CD20 protein, the target for rituximab, may be a potential cause of poor response to R-CHOP therapy.

1.6.2 Hypothesis 2

Given the essential roles of *MYC* and *BCL2* in determining B cell fate, de-regulation of these oncogenes though translocations may be associated with a poor clinical outcome in DLBCL.

1.7 Aims and thesis outline

The thesis consists of 4 manuscripts that address both hypotheses outlined above.

1.7.1 Aim 1: To determine the incidence and prognostic significance of CD20 mutations in DLBCL

Chapter 2 describes the incidence of mutations involving the *MS4A1* gene in a large unselected cohort of DLBCL samples taken prior to and following R-CHOP therapy. It also correlates the presence of *MS4A1* mutations with measurement of CD20 protein expression by FCM and IHC.

1.7.2 Aim 2: To determine the prognostic significance of CD20 protein expression in DLBCL

Chapter 3 demonstrates that neoplastic B cells in DLBCL have heterogeneous expression of both CD20 and CD19 expression as determined by FCM and that the patterns of expression are associated with clinical outcome.

1.7.3 Aim 3: To determine the prognostic significance of genomic rearrangements in *BCL2* and *MYC* in DLBCL

Chapter 4 is a study of the cytogenetic and clinical factors associated with survival in lymphomas that harbour concurrent translocations in *BCL2* and *MYC*. It sets the stage for the hypothesis and aims in chapter 5. Chapter 5 is an international collaborative study investigating the incidence and prognostic impact of *BCL2* and *MYC* translocations in a DLBCL cohort treated uniformly with R-CHOP. It also correlates the presence of *MYC* translocations with *MYC* mRNA expression.

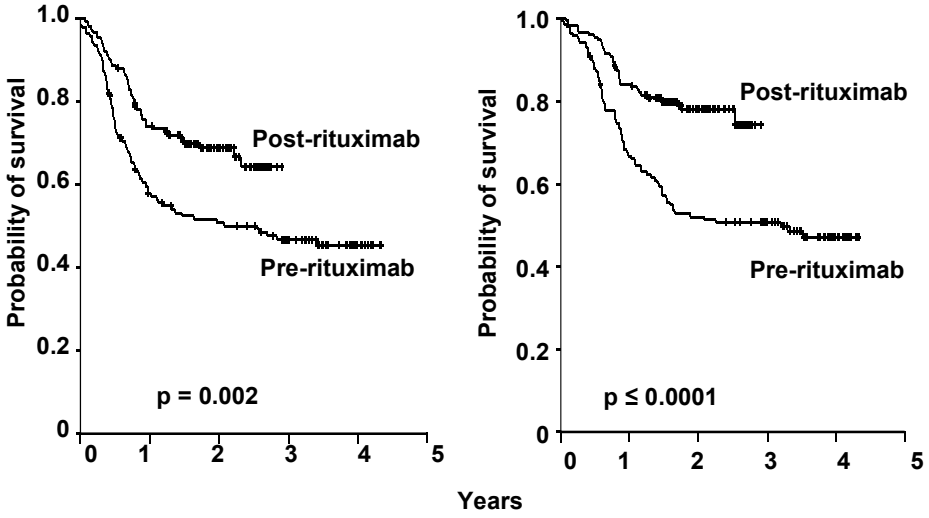

Figure 1.1: Progression-free survival (left) and overall survival (right) by treatment era in BC, all patients (n=292)

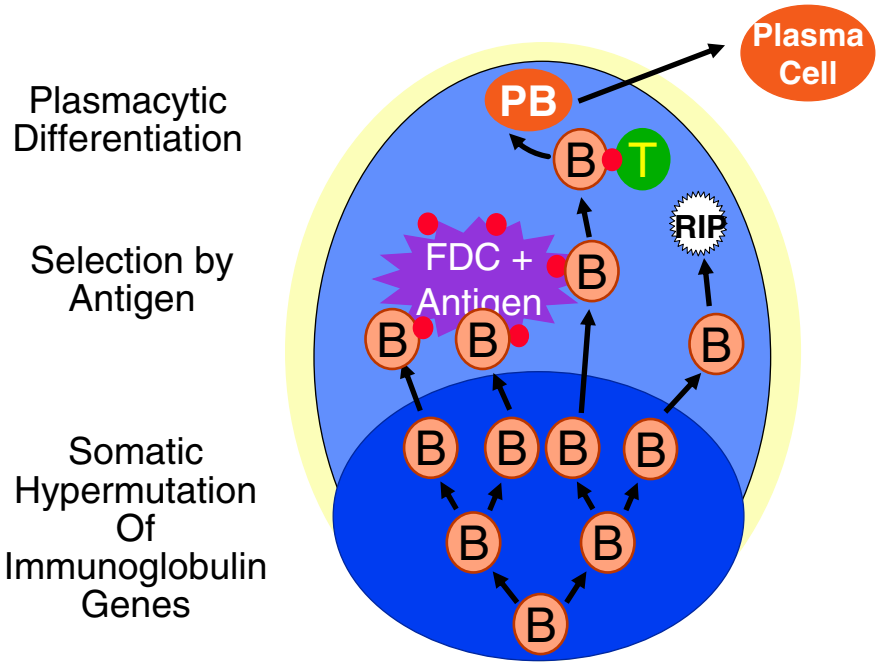

Figure 1.2: The germinal centre reaction

Abbreviations: B, B lymphocytes; T, T lymphocytes; RIP, B lymphocyte undergoing apoptosis; FDC, follicular dendritic cell; PB, plasmablast

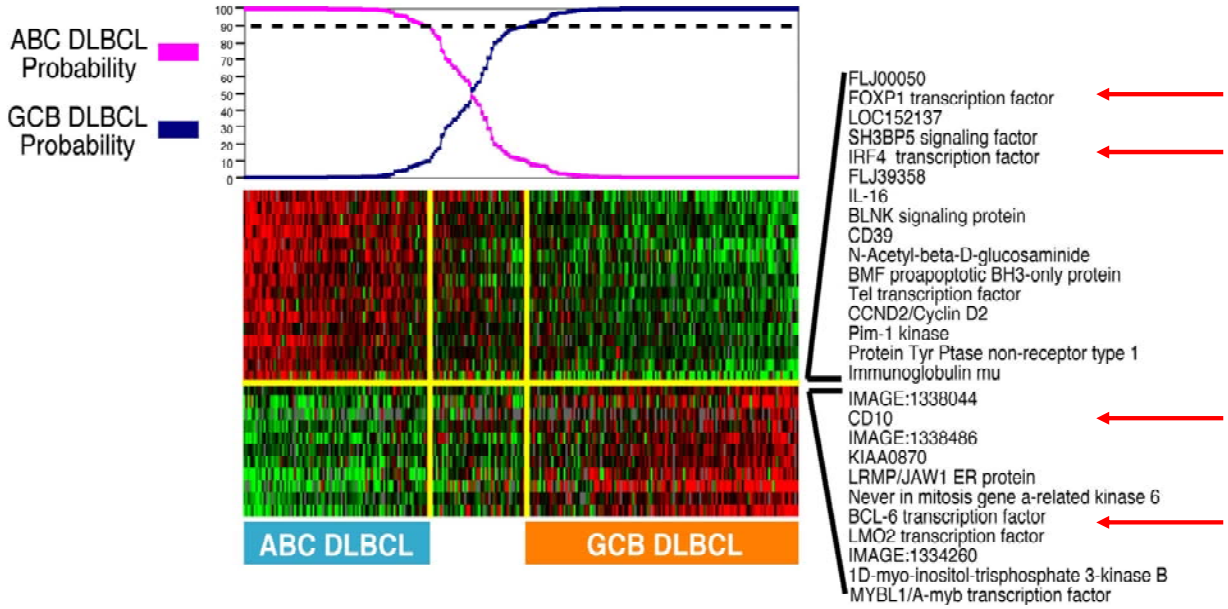

Figure 1.3: A Bayesian classifier to estimate the probability that a lymphoma is ABC versus GCB subtype of DLBCL

Arrows depict genes used in the classifier by immunohistochemistry. Adapted from Wright, PNAS 2003 (ref 34)

1.8 References

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Chapter 2: CD20 mutations involving the rituximab epitope are rare in diffuse large B cell lymphomas and are not a significant cause of R-CHOP failure*

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2.1 Introduction

Rituximab is a chimeric monoclonal antibody targeting the CD20 antigen on B lymphocytes [1]. The addition of rituximab to multi-agent chemotherapy has improved survival in patients with DLBCL [2,3]. Because of this, there has been great interest in determining the role of CD20 in the pathogenesis of lymphomas and its function in normal B cells. The CD20 antigen is a membrane bound protein that contains four trans-membrane domains and a large extra-cellular loop [4]. Two amino acid sequences, ANPS and YCYSI at positions 170 to 173 and 182 to 185, were recently determined to be the critical binding sites for rituximab [5,6]. The cytotoxic effects of rituximab, such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and direct induction of apoptosis require that the primary event be the binding of rituximab to CD20 [7]. The rituximab epitope and part of the third and fourth trans-membrane domain are all included in exon 5 of the *MS4A1* gene. We sequenced exon 5 of *MS4A1* in primary DLBCL samples taken at diagnosis and relapse to determine the frequency and clinical significance of mutations at that site in R-CHOP treated patients. We correlated the presence of mutations with protein expression using immunohistochemistry (IHC) and flow cytometry (FCM).

2.2 Methods

Patient Selection:

Patients diagnosed with DLBCL according to the World Health Organization (WHO) criteria and who had tissue available at the time of diagnosis and/or

relapse between March 1st 2001 and December 1st 2006 were included in this study [8]. Baseline clinical characteristics, treatment regimen and clinical outcome were recorded for all patients. This research was approved by the University of British Columbia and BCCA research ethics board and is in accordance with the Declaration of Helsinki.

Sequencing the MS4A1 gene:

277 patients had initial biopsy tissue and 18 patients had relapse biopsy tissue available for sequencing. 10 patients had paired samples taken both at diagnosis and relapse. DNA was extracted using ALL PREP DNA/RNA mini kit (Qiagen) and PureGene DNA purification kit (Gentra) for frozen tissue and formalin fixed paraffin embedded tissue (FFPET) respectively.

We amplified exon 5 with the following PCR primers: 5'-*TGTAAAACGACGGCCAGT*TTGGAATTCCCTCCCAGATT-3' and 5'-*CAGGAAACAGCTATGAC*GGATCCAGAGTTCATGCTCA-3'. -21M13F and M13R sequencing tag extensions (italics) were incorporated at the 5' ends of the forward and reverse primers, respectively, to allow sequencing with standardized M13 primers and protocols[9]. The 431 base pair PCR product was purified with AMPure magnetic beads (Agencourt Bioscience Corporation) and bi-directionally sequenced using BigDye® Terminator v3.1 (Applied Biosystems) and an ABI 3730 XL sequencer. The forward and reverse sequence reads were assembled together and analyzed using PolyPhred [10] and displayed using Consed [11], or

analyzed using Mutation Surveyor (SOFTGENETICS, PA, USA). Mutations were considered present if they were found in both forward and reverse reads.

Determining CD20 protein expression:

CD20 protein expression by IHC was determined on all samples using the L26 antibody on FFPET (Dako). CD20 protein expression was determined by FCM on 227 and 5 samples taken at diagnosis and relapse, respectively. Tumour cell suspensions were created by disaggregating cells from fresh tissue and suspending them in phosphate buffer (Dulbecco's PBS, Stem Cell Technologies) at a concentration of 10^7cells/ml. Cells were incubated at 4^oC for 30 minutes with antibodies conjugated to either fluorescein isothiocyanate (FITC), phycoerythrin (PE) or phycoerythrin-Cy5 (PE-Cy5). The following antibody combinations were used: 10 μl of a combination of anti- kappa-FITC, anti-lambda-PE and anti-CD19-PE-Cy5 and 10 μl of each anti-CD10-FITC, anti-CD11c-PE, anti-CD20-PE-Cy5 (clone B9E9, B1 epitope). Cells were then treated with 250 μl of Opti-Lyse C and washed once with IsoFlow sheath fluid prior to FCM analysis on a Beckman Coulter Cytomics FC500. 500 x 10^6 cells from 55 samples of normal peripheral blood lymphocytes (PB) were used as a "control" and were treated using the same method except that a 15 minutes incubation time was used. All reagents were purchased from Beckman Coulter except for the antibodies against CD19 and light chains which were purchased from Dako. A minimum of 5000 events were analyzed using Flow Jo software version 8.7.1. The mean fluorescence intensity (MFI) of CD20 in the tumour cells and benign PB lymphocytes were

recorded. The tumour content was defined as the percent cells co-expressing CD19 and the tumour specific light chain in the total live cell gate, determined using forward scatter and side scatter.

2.3 Results

264/277 (95%) and 15/18 (83%) the DLBCL samples taken at diagnosis and relapse were successfully sequenced. The patient's clinical characteristics are found in Table 2.1 and were similar to those reported by Sehn et al.(2005), suggesting that our results were not biased by a dependence on frozen tissue and are representative of the DLBCL patient population in British Columbia, Canada [3]. The majority of the patients had nodal disease with a minimum of 80% tumour, sufficient tumour cell content that if mutations were present, they would be detectable by sequencing. Lymphomas progressed or relapsed in 24% of the patients following R-CHOP immuno-chemotherapy.

One of 264 samples (0.4%) taken at diagnosis showed a 13 base pair (bp) heterozygous deletion at position IVS5 (+8) in intron 5. This region is not known to contain regulatory elements or alternate splice sites. Germline DNA was not available to determine if this represented a polymorphism or a somatic mutation. Clinically, this patient achieved a complete response to R-CHOP and remains in remission more than 2 years after diagnosis. Only 1/15 (6%) sample taken one month after R-CHOP showed a CD20 mutation. This case had a heterozygous 4 bp deletion (TAAT) at nucleotide position 353-356 which predicted for a

premature termination at amino acid position 121, well before the critical ANPS binding site (see Figure 2.1). Unfortunately, no pre-treatment biopsy was available to determine if the mutation was present at diagnosis. No single nucleotide polymorphisms (SNPs) were detected in exon 5 of the CD20 gene.

Protein expression of CD20 was assessed in all cases by IHC using the antibody L26 used routinely in most clinical laboratories[12]. This antibody recognizes a cytoplasmic epitope of the CD20 antigen, distinct from the rituximab binding site [13]. All of the initial 277 samples had uniform and bright CD20 protein expression. Three patients had CD20 negative biopsies at the time of relapse where the initial biopsy was CD20 positive. Two of these samples contained malignant cells with different phenotypes; large tumour cells that were clearly CD20 negative while others were positive (see Figure 2.2). The clinical outcome of these patients was poor. Two patients died within 6 months of relapse and the other is receiving salvage chemotherapy in preparation for an autologous stem cell transplant 4 months after relapse. Interestingly, the sample taken at relapse showing a CD20 mutation that predicted for a severely truncated protein and loss of the extra-cellular domain, showed strong CD20 protein expression by IHC and FCM (see Figure 2.1b and c). The mean CD20 MFI of this mutated sample was 49.7 compared to the mean CD20 MFI of 55.97 (standard deviation,105) for 80 DLBCL samples that were analyzed during the same time frame where all instrument settings and analysis protocols remained constant (see inset of Figure 2.1b). The mean CD20 MFI of normal PB lymphocytes during this same time

frame was 238 (standard deviation, 105). One patient had a sample taken at relapse that had strong CD20 expression by IHC but weak CD20 expression by FCM (CD20 MFI <10). However, the initial biopsy on this patient had similar CD20 expression by FCM (MFI<10) prior to rituximab exposure.

2.4 Discussion

The recent identification of the rituximab binding site prompted us to determine the frequency of CD20 mutations at that site as a possible cause of primary R-CHOP resistance [5]. We show that the frequency of mutations coding for the extra-cellular domain of the CD20 gene is extremely low in both *de novo* and relapsed DLBCL and is not a significant cause of R-CHOP treatment failure. Indeed, the only mutation found in a relapse biopsy was heterozygous and did not result in a change in CD20 protein expression as determined by IHC and FCM, implying that the normal allele may be sufficient to support a normal protein expression level. Furthermore, recent work by Czuczman et al. confirms that mutations in the CD20 gene are not the cause of decreased CD20 protein expression in rituximab resistance cell lines [14].

Mutations at other sites on CD20 could also potentially lead to R-CHOP resistance but were not addressed in this study. In an unpublished study of CD20 mutations in 50 non-Hodgkin's lymphoma (NHL) samples, which included different lymphoma sub-types and samples taken at relapse, investigators noted a 22% incidence of mutations with cytoplasmic mutations occurring four times

more frequently than those in the extra-cellular domain [15]. Mutations at residues 219 to 252 could prevent the formation of lipid rafts in response to binding of rituximab [16,17]. Cross-linking of CD20 monomers into lipid rafts may be important in enhancing CDC activity and apoptosis [18]. Even if one assumes that these mutations are clinically meaningful, the incidence of *de novo* CD20 mutations is still too low to justify a screening strategy to identify potential rituximab non-responders.

Our finding of reduced CD20 protein expression detected by IHC in three samples taken at relapse indicates that CD20-negative relapses can occur and may be more common than previously described by Davis et al. [19]. Indeed, since then other case reports and case series have been described but in variably treated patients with different lymphoma subtypes [20-22]. Tissue obtained from patients with primary DLBCL at the time of relapse is rare because most patients undergo fine needle aspiration to confirm relapse or have no biopsy. Thus the true incidence of CD20 negative relapses is unknown. In our cohort, 3 patients who initially had CD20 positive DLBCL had malignant clones in their samples at relapse that were clearly CD20 negative. Unexpectedly, two of these patients showed clonal heterogeneity in which only a subset of cells were CD20 negative, a phenotype that to our knowledge has never been described before. Weak CD20 protein expression by FCM but strong CD20 staining by IHC was seen in 13 and 1 biopsies taken at diagnosis and relapse, respectively. Recent rituximab exposure can account for apparently CD20 negative B cells by FCM as both

rituximab and B1 compete for the same binding site [23]. However, this is not the case in our study. We can not exclude that other genetic alterations in the CD20 gene or post transcriptional regulation of CD20 may have occurred. Aneuploidy and translocations are also common in NHL but unlike chronic lymphocytic leukemia, deletions at chromosome 11q21 in DLBCL are infrequent as assessed by array comparative genomic hybridization [24]. Interestingly, lymphomas progressed or relapsed in 7/13 patients with weak CD20 expression on their primary biopsy by FCM, but these small numbers preclude meaningful conclusions. Thus, the CD20 antigen may play a role in the pathogenesis of DLBCL, but our data suggest that mutations in the rituximab epitope do not occur with sufficient frequency to account for a meaningful proportion of the observed treatment failures.

Clinical characteristics	Initial diagnostic biopsies n = 277 (%)	Biopsies at relapse n = 18 (%)
Median age, years	64	60
Male sex	171 (62)	12 (67)
PS > 1	110 (40)	6 (33)
LDH > normal	161 (58)	10 (55)
Extranodal sites > 1	100 (36)	7 (39)
Stage III/IV	177 (64)	14 (78)
IPI score at diagnosis:		
0	33 (12)	0 (0)
1-2	149 (54)	10 (55)
3-4-5	95 (34)	8 (45)
Pathology of biopsy:		
DLBCL	255 (92)	16 (89)
PMBCL	22 (8)	
FL		2 (11)
Site: nodal	219 (79)	10 (55)
extranodal	58 (21)	8 (45)
Flow cytometry:	227 (82)	5 (28)
> 50% tumour content	161 (71)	4 (80)
CD20 Fluorescent intensity <10	13 (5)	1 (20)
CD20 protein expression by IHC		
Negative	0	1 (6)
Heterogeneous		2 (12)
DLBCL relapse or progression after R-CHOP	66 (24)	18 (100)

Table 2.1: Patient characteristics
Abbreviations: PS: ECOG performance status; LDH, lactate dehydrogenase; IPI, International Prognostic Index; DLBCL, diffuse large B cell lymphoma; PMBCL, primary mediastinal B cell lymphoma; FL, follicular lymphoma; IHC, immunohistochemistry.

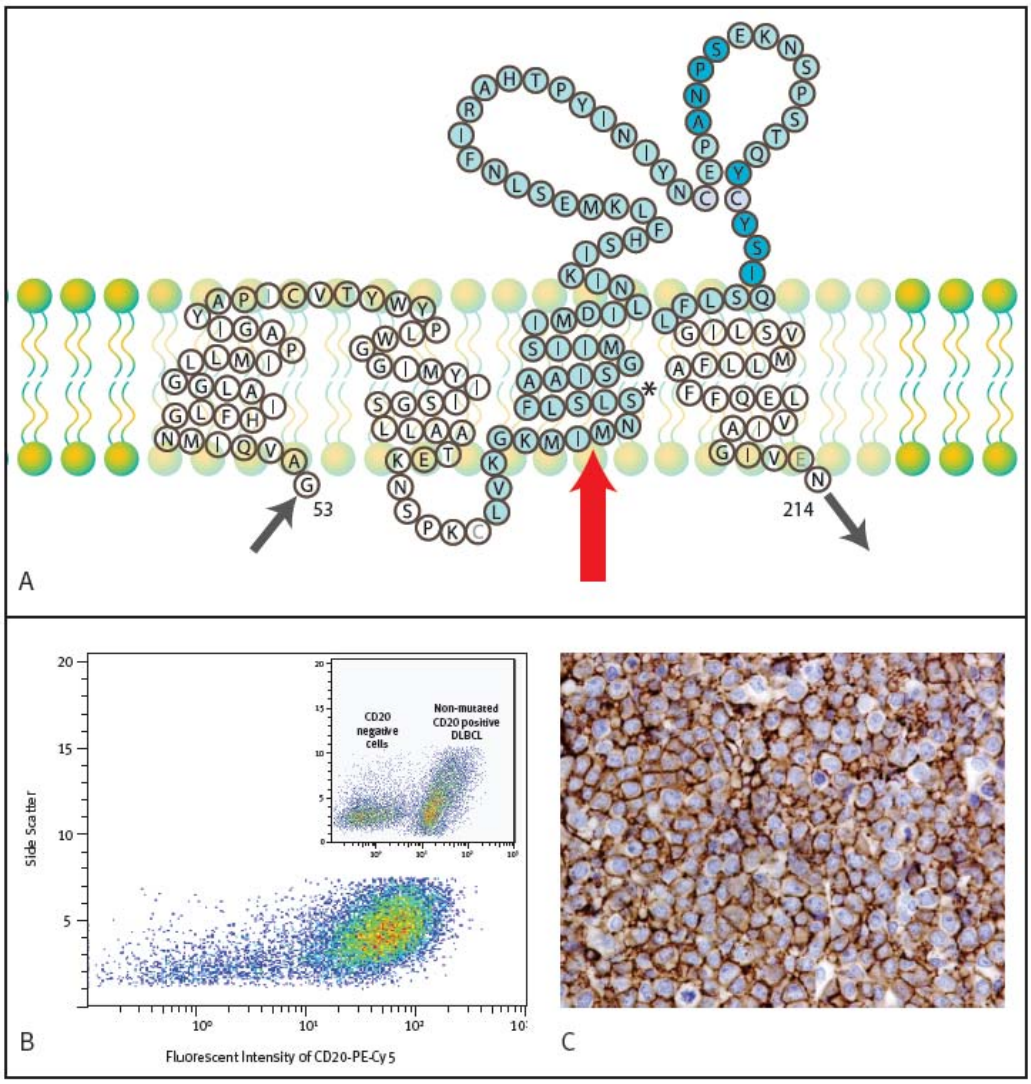

Figure 2.1: CD20 expression in a DLBCL sample taken at relapse containing a 4 base pair deletion at nucleotide position 353-356

Box A: Representation of the amino acid sequence coding for the trans-membrane portion of the CD20 protein (adapted from Binder et al.[5])
Light blue amino acids are contained within the sequenced region of exon 5 of the MS4A1 gene and dark blue amino acids represent the rituximab epitope brought together by a disulfide bond at two cysteine residues. The red arrow represents the location of the 4 base pair deletion leading to premature termination at amino acid 121 (highlighted with a star).
Box B: Bright CD20 protein expression by flow cytometry in the mutated sample; inset: CD20 expression of a non-mutated DLBCL sample analyzed during the same time frame demonstrating CD20 fluorescent intensity of benign non-B cells (CD20 negative) and tumour cells (CD20 positive) within the sample.
Box C: Bright CD20 protein expression by immunohistochemistry in the mutated sample (L26 antibody)

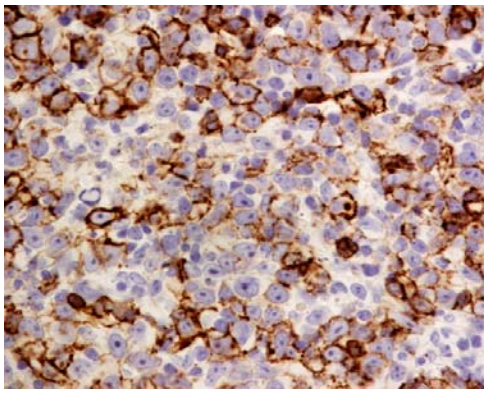

Figure 2.2: Heterogeneous CD20 protein expression by immunohistochemistry in a DLBCL sample taken at relapse following R-CHOP therapy

2.5 References

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Chapter 3: Diffuse large B cell lymphoma: reduced CD20 expression is associated with an inferior survival*

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3.1 Introduction

Diffuse large B cell lymphoma (DLBCL) represents 40% of the non-Hodgkin lymphomas (NHL) and expresses the classical B cell markers found on normal B lymphocytes, that is, CD19, CD20 and CD79a[1]. The CD20 antigen is a membrane-bound protein that is thought to play a role in B cell activation, differentiation and cell cycle progression[2,3]. Rituximab (R) is a monoclonal antibody directed against the CD20 antigen and its addition to cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) has dramatically improved the survival of patients with DLBCL [4,5]. However, not all patients are cured by this primary therapy and insight into the mechanisms of treatment failure may guide the development of better therapy in the future.

CD20 protein expression, as determined by flow cytometry (FCM), is very heterogeneous between and within different lymphoma subtypes[6]. For instance, CD20 expression in small lymphocytic lymphoma (SLL)/chronic lymphocytic leukemia (CLL) is usually lower (dim CD20) than in follicular lymphoma (FL) and this difference may correlate with clinical responses to rituximab[6]. In the pivotal trial conducted by McLaughlin *et al.*, only 13% of patients with SLL/CLL compared to 60% of patients with FL ($p < 0.01$) responded to rituximab[7]. Olejniczak *et al*. found that CD20 expression in DLBCL also showed marked variability and that some samples had “dim” CD20, similar to that of SLL/CLL[6]. We hypothesized that such patients would have an inferior response to R-CHOP

compared to patients with “bright” CD20 expression on their lymphoma cells. The goal of this study was to determine the frequency of reduced (dim) CD20 expression relative to CD19 expression in DLBCL samples at diagnosis and to correlate this finding with clinical outcome in patients treated with CHOP +/- R. Furthermore, we compare CD20 protein expression by FCM to CD20 expression determined by immunohistochemistry (IHC).

3.2 Methods

Patient Selection:

Patients with *de novo* DLBCL, diagnosed by experienced hematopathologists (RDG and MC) according to the World Health Organization (WHO) criteria who had FCM analysis performed on their diagnostic biopsies between 1997 and 2007 were included in this study[1]. Patients were greater than 18 years old, HIV-negative and treated with curative intent with CHOP +/- R. Their baseline clinical characteristics, including the international prognostic index (IPI) variables, pathology of their staging bone marrow and clinical outcomes were recorded. All patients treated with CHOP-R at the British Columbia Cancer Agency were required to have CD20-positive DLBCL by IHC. Ethical approval to conduct this retrospective review was granted by the University of British Columbia - British Columbia Cancer Agency Research Ethics Board in accordance with the Declaration of Helsinki.

Flow cytometry:

Monoclonal antibodies:

Cell suspensions from freshly disaggregated lymph node biopsies were stained according to the manufacturer's recommendations with monoclonal antibodies conjugated to either fluorescein isothiocyanate (FITC), phycoerythrin (PE) or phycoerythrin-Cy5 (PE-Cy5). The routine diagnostic panel comprised the following 7 tubes. Tube 1 contained anti-CD45-FITC, anti-CD14-PE and anti-CD19-PE-Cy5. Tube 2 contained isotype controls IgG1-FITC, IgG1/IgG2a-PE and IgG1-PE-Cy5. Tube 3 contained anti-CD10-FITC, anti-CD11c-PE, anti-CD20-PE-Cy5. Tube 4 contained anti-CD5-FITC, anti-CD19-PE and anti-CD3 PE-Cy5. Tube 5 contained anti-CD7-FITC, anti-CD4-PE, anti-CD8-PE-Cy5. Tube 6 contained anti-FMC7-FITC, anti-CD23-PE and anti-CD19-PE-Cy5. Tube 7 contained anti-kappa-FITC, anti-lambda-PE and anti-CD19-PE-Cy5. The anti-CD20 antibody was directed against the B1 epitope, clone B9E9. All antibodies were obtained from Beckman Coulter (Mississauga, Ontario) except CD23, kappa, lambda and CD19-PE-Cy 5 (in tube 7), which were obtained from Dako (Mississauga, Ontario).

Cell preparation:

The cell suspensions were generated by disaggregating cells from fresh tissue and suspending them in phosphate buffer solution (Dulbecco's PBS; Stem Cell Technologies) to a lymphocyte concentration approximating 10^7/ml. Cell concentration and viability was assessed using Trypan blue exclusion dye

(Invitrogen). 500,000 live cells were stained with the appropriate antibody combinations (see above) and incubated at 4^0C for 30 minutes. Cells were treated with 250 μl of Opti-Lyse C containing 1.5% formaldehyde (Beckman Coulter) to deplete red cells and fix the lymphocytes. The remaining cells were then washed once with IsoFlow sheath fluid (Beckman Coulter) prior to FCM analysis. Peripheral blood (PB) lymphocytes taken from 67 patients without lymphoma were counted using the Bayer Advia 120 hematology system cell counter and diluted in PBS to a concentration of 1 to 10 x 10^9/ml. 500 x 10^6 cells were then treated using the same method as described above except that PB lymphocytes were incubated with antibody combinations at room temperature for 15 minutes.

Flow cytometry analysis:

Quantitative fluorescence analysis was performed using a Beckman Coulter Cytomics FC500 equipped with a single 488 nm argon laser source. FITC/PE/PE-Cy5 emission was collected in FL1/2/4 channels using 525/575/675 nm bandpass filters, respectively. Daily instrument calibration was performed using Flow-Set/Flow-Check beads (Coulter, Mississauga).

We noted that the voltage settings of the cytometer were changed significantly twice between 1997 and 2007 as analysis protocols evolved in the lab, however, within the three time windows (1997 – 2002 (Feb), 2002 (March) – 2004 (Nov), 2004 (Dec) – 2007), these settings remained constant. Thus the MFI for specific

antigens in samples studied within each time window could be compared to each other.

Data analysis:

List mode files were analyzed using FLOW JO software version 8.7.1. A minimum of 5000 events were analyzed for all gated populations presented. Live cells were gated using forward and side scatter criteria. The MFI, variance and standard deviation were recorded for each cell population of interest. The samples within each of the three time frames (1997 - 2002, 2002 - 2004, 2004 - 2007) were then rank ordered by MFI. In each of the three time frames, a natural bimodal distribution was apparent which allowed an MFI cut off value to be defined, separating the samples into “dim” vs “bright” subpopulations. Staining for the T cell marker CD3 allowed discrimination between “dim” CD20 B cells and CD20-negative T cells. The CD19 MFI distribution was also rank ordered and “dim” CD19 defined in a similar fashion as “dim” CD20. A sample was considered CD5-positive if the CD19 positive events (determined in tube 1) also stained positive for CD5 (CD5+). The threshold for calling a CD5-positive event was determined using the CD5 fluorescent intensity of T cells which co-expressed CD3 and CD5 in tube 4.

Immunohistochemistry:

CD20 protein expression using formalin fixed paraffin embedded tissue (FFPET) was assessed using routine methods of staining (Ventana) with the L26 antibody

(Dako) directed against a cytoplasmic epitope of the CD20 antigen[8]. Cyclin D1 (Dako) staining was performed on all cases that co-expressed CD19 and CD5 by FCM.

Determination of DLBCL subtypes:

69 patients had sufficient tissue available at diagnosis that a portion of the biopsy was frozen in liquid nitrogen and stored at -80^{o}C while the remaining tissue was used for FCM. 200 μm of this fresh frozen tissue was sectioned in a cryostat and total RNA was extracted using the ALL PREP kit (Qiagen). Total RNA was reversed transcribed (one cycle) and hybridized to U133-2 Plus arrays according to the manufacturer's protocol (Affymetrix). CEL files were normalized using robust multi-chip analysis (RMA)[9]. Cell of origin (COO) was calculated using model scores for activated B cell type (ABC) and germinal B cell type (GCB) derived from the 100 gene model described by Dave *et al.* and the Bayesian formula described by Wright *et al*.[10,11]. A subset of 61 patients had FFPET available for staining for Bcl-6 protein, MUM1 and CD10. COO was determined as GCB and non-GCB according to Hans criteria[12].

Sequencing of exon 5 of the MS4A1 (*CD20) gene:*

Fifteen samples that were considered discordant CD20 ("dim" CD20 and "bright" CD19) had sufficient remaining frozen tissue to allow extraction of DNA using the ALL PREP kit (Qiagen). We amplified exon 5 of *MS4A1* with the following PCR primers: 5'-*TGTAAAACGACGGCCAGT*TTGGAATTCCCTCCCAGATT-3' and 5'-

*CAGGAAACAGCTATGAC*GGATCCAGAGTTCATGCTCA-3'. -21M13F and M13R were used as sequence tag extensions (italics) to facilitate sequencing with standardized M13 primers[13]. The purified 431 base pair PCR product was bi-directionally sequenced using BigDye® Terminator v3.1 (Applied Biosystems) and an ABI 3730 XL sequencer (Applied Biosystems). The forward and reverse sequence reads were assembled together and analyzed using PolyPhred and displayed using Consed[14,15].

Statistical Analysis:

Univariate survival analysis was performed using the log rank test and Kaplan Meier method (SPSS software, version 11). The Cox proportional hazard model was used to determine the relationship between survival and the known covariates in this study. The Fisher's exact test was used to determine the association between CD20 expression and CD5 expression. Two sided p-values of 0.05 were considered significant.

3.3 Results

A total of 272 patients with newly diagnosed DLBCL had CD20 expression by FCM performed on their primary biopsy and had complete clinical information to be included in this analysis. The baseline clinical characteristics were similar in both CHOP and R-CHOP treated patients (see Table 3.1). R-CHOP treated patients had a superior overall survival (OS) than CHOP treated patients ($p = 0.03$) over a median follow time of 3.2 and 6.0 years, respectively. Thus both

patient groups were analyzed separately when assessing the association of CD20 expression with clinical outcome. Each sample was re-analyzed for CD3, CD19, CD20 and FMC7 expression. We found that the tumour content across samples was variable and contaminating T cells represented a significant portion of the cells present. The average T cell content was 37% and one third of the samples had a T cell content of > 50%.

CD20 expression by FCM is heterogeneous

The CD20 MFI varied considerably within each of the three time windows during which instrument settings and laboratory protocols remained constant. This heterogeneity was very similar to that observed by Olejniczak *et al.* who used a more sensitive quantitative assay for determining CD20 density on DLBCL[6]. Figure 3.1a demonstrates the distribution of MFI in the DLBCL samples analyzed from 2004 - 2007. Two distinct groups could be identified based on CD20 expression. Thirteen samples (16% of the group) had a very low MFI (range 0.85 to 11.57) and 67 samples had higher MFIs (range 23.9 to 450). The first group was defined as having “dim” or reduced CD20 expression whereas the remaining samples were considered “bright” because their CD20 expression was closer to that of normal PB lymphocytes, as seen in Figure 3.1c. The mean MFI of CD20 of these 67 samples was 93.9 compared to 238 for normal PB lymphocytes. T cells, which were present in all of the samples, served as an internal negative control and had a mean MFI of 0.38. Due to the staining and acquisition protocols employed in the first two time windows (1997 - 2002 and

2002 - 2004), the dynamic range of CD20 expression was compressed relative to the 2004 - 2007 period. As such, a definitive "trough" could not be identified to demarcate "dim" from "bright" cases, despite the obvious presence of a "dim" subset. In order to define a cut-off MFI to segregate "dim" from "bright" in this situation, we made the assumption that the fraction of "dim" vs "bright" cases should be similar between the three time frames, and arbitrarily defined the dimmest 16% of cases (ranked by MFI) to be "dim" and the rest as "bright".

CD19 expression by FCM is heterogeneous

CD19 expression was also very heterogeneous and showed similar distribution patterns to CD20 (see Figures 3.1b and 1d). This was also true for FMC7, an epitope of CD20 (data not shown)[16,17]. Interestingly, one sample showed at least 3 populations of CD19 positive cells displaying different intensities of FMC7, suggesting that clonal populations with different CD20 expression can exist within the same tumour (see Supplemental Figure 3.1). All but one sample with "dim" CD20 expression also had "dim" expression for FMC7. However, 12% of the "dim" FMC7 samples were "bright" for CD20. Overall, 4 major groups were defined based on the pattern of their CD19 and CD20 expression (see Table 3.3). These groups will be referred to as concordant bright, concordant dim, discordant CD19 ("dim" CD19 and "bright" CD20) and discordant CD20 ("dim" CD20 and "bright" CD19).

CD20 expression by FCM is more sensitive than IHC

We then compared CD20 protein expression determined by FCM to that obtained by IHC. The B1 antibody used in most clinical FCM laboratories targets the same critical amino acid sequence on the extracellular CD20 epitope as rituximab whereas the L26 antibody used routinely on FFPET, targets the cytoplasmic portion of CD20[8,18]. In total, 16% (n = 43) of the DLBCL samples were "dim" CD20 (including both discordant CD20 and concordant dim) by FCM but only 3 cases were CD20 negative by IHC. This relative low frequency of CD20 negative biopsies reflects that CD20 expression by IHC was a requirement in order to be treated with rituximab at our institution. Thus these 3 negative biopsies were in CHOP treated patients only. Dots plots and histology sections of representative "dim" CD20 and "bright" CD20 samples in Figure 3.2 demonstrate that the one log intensity difference in CD20 expression detected by FCM could not be detected using routine IHC. Side scatter, representing internal cellular complexity, was the best parameter to distinguish the CD20 negative T cells from the "dim" CD20 malignant B cells.

Reduced CD20 expression is associated with an inferior survival

Reduced CD20 expression ("dim" CD20) in primary DLBCL was associated with a median OS of 1.2 years and 3 years for the "dim" CD20 versus median survival not reached in the "bright" CD20 group in CHOP and R-CHOP treated patients, respectively (see Figures 3.3a and b). Dichotomizing the data according to CD20 and CD19 expression, we found that patients whose biopsies were

discordant CD20 (i.e. “dim” CD20 but “bright” CD19) had the worst OS compared to patients whose biopsies were concordant dim or concordant bright, irrespective of treatment regimen (see Figures 3.3c and d). Interestingly, the poor prognostic effect of discordant CD20 was also seen in the CHOP treated patients suggesting that CD20 expression correlates with the cellular biology of the malignant lymphocytes and that the CD20 antigen is important beyond merely serving as a rituximab target. Indeed, 8/10 (87%) and 22/35 (63%) of patients with discordant CD20 eventually relapsed after CHOP and R-CHOP suggesting that these were very high-risk patients even when rituximab was introduced into the treatment regimen. The discordant CD19 group had a slightly inferior survival compared to the concordant bright group in CHOP treated patients but this non-significant negative prognostic effect disappeared when rituximab was included in the treatment regimen. Although all the discrepant CD20 samples were also “dim” or negative for FMC7, FMC7 expression alone was not correlated with survival. Thus, in DLBCL, a reduced CD20 expression was associated with an inferior survival if CD19 expression was “bright” (discordant CD20).

Discordant CD20 expression is associated with CD5 expression and Bcl-2 expression

The clinical and pathological characteristics of patients whose biopsies had discordant CD20 expression were slightly different than the other groups. These patients tended to present more often with advanced-stage disease and higher

IPI scores. In addition, 11 (31%) of these patients had biopsies that showed co-expression of CD19 and CD5 (CD5+). Importantly, these were not patients with "Richter's transformation" because their staging bone marrow biopsies did not contain CLL nor were they cases of misdiagnosed mantle cell lymphomas because the biopsies were all negative for cyclin D1[19]. Given that CD5+ DLBCL has been previously shown to be associated with an inferior survival in CHOP and more recently in R-CHOP treated patients, we determined the association of CD5 co-expression on B cells with clinical outcome[20,21]. Indeed, CD5+ DLBCL was associated with an inferior survival in both CHOP and R-CHOP treated patients ($p = 0.008$ and $p = 0.008$, respectively) (see Figure 3.4a and Figure 3.4b). Similar to the discordant CD20 group, these patients also presented with advanced-stage disease and higher IPI scores but unlike previous reports, this was predominantly seen in older men. However, 13/24 (54%) biopsies that were CD5+, were also discordant for CD20. Thus, CD5+ DLBCL is highly associated with reduced CD20 expression ($p = 0.0001$).

Eighty one percent and 83% of biopsies in the discordant CD20 and CD5+ groups were also positive for Bcl-2 protein, respectively, which, as expected, correlated with a significantly inferior survival in CHOP but not R-CHOP treated patients ($p = 0.01$ and $p = 0.9$, respectively). Other parameters such as CD10 expression and CD4/CD8 ratio were not associated with OS or discordant CD20 status.

Discordant CD20 expression remains a predictor of outcome on multivariate analysis

When CD5 status, discordant CD20 and IPI were included as covariates in a Cox regression analysis in R-CHOP treated patients, only IPI and discordant CD20 remained as statistically significant predictors of overall survival (IPI, p = 0.007; discordant CD20, p = 0.002). Thus the negative prognostic effect of CD5+ appears to result from its association with reduced CD20 expression and high-risk clinical features.

Reduced CD20 expression is not caused by mutations in exon 5 of the *MS4A1* gene

To explain the discrepancy between dim CD20 by FCM and bright CD20 by IHC observed in 94% of the discordant CD20 samples, we sequenced exon 5 of the *MS4A1* gene which codes for the extracellular loop of the CD20 protein. Mutations at the critical ANPS and YCYSI motifs at amino acids 170-173 and 182-185 have, in previous *in vitro* studies, sufficiently altered the quaternary structure of CD20 to affect the binding affinity of B1 and other CD20 antibodies[22]. In this study, 15 of the discordant CD20 cases were successfully sequenced and no mutations were detected. DLBCL subtype defined by gene expression profiling has been shown to be associated with OS in CHOP and more recently R-CHOP treated patients[23-25]. Thus, we determined if there was an association between discordant CD20 and cell of origin in 18 discordant CD20 biopsies and 13 CD5+ biopsies. We found a similar proportion of GCB and ABC subtypes in

the CD5+ group but a relatively high proportion of the ABC subtype in the discordant CD20 group (12/18) (see Table 3.2). Thus, cell of origin may be a confounding factor in the prognostic effect of discordant CD20 expression.

3.4 Discussion

We show that CD20 expression in DLBCL is heterogeneous and that at least 16% of cases (3% concordant dim and 13% discordant CD20) have reduced levels of CD20 similar to what is observed in a sizable proportion of cases of SLL/CLL. The prognostic significance of CD20 expression is contentious in other lymphoma subtypes and to our knowledge, has never been specifically examined in DLBCL[6,26-29]. We demonstrate that patients who have reduced CD20 expression but bright CD19 expression (discordant CD20) on their biopsies taken at diagnosis have a markedly inferior OS following treatment with CHOP +/- R, independent of the IPI.

Quantitative measurements of fluorescence intensity using microbead standards would be considered the "gold standard" in determining the number of antigens on specific cell populations of interest[30]. Recently, three such assays were tested in CLL and they were found to be very reproducible at assessing quantitative CD20 antigen expression[31]. We did not use such methods to assess antigen expression in our study, possibly accounting for some of the variability we encountered. However, our FCM data were accrued on a single instrument with constant configuration for the entire cohort of samples included in this study

and with only two significant alterations in voltage settings over a 10 year period. All other instrument parameters were held constant for the entire decade, thus allowing the analysis of hundreds of samples on a consistent instrument platform. The results of this study provide sufficient evidence that the use of FCM with proper calibration standards should be utilized to study more B-cell neoplasms, including SLL/CLL, whenever patients are candidates for anti-CD20 immunotherapy.

Importantly, the immunofluorescence assay by FCM used in our study was more sensitive at detecting differences in CD20 antigen expression than IHC. Indeed, IHC missed 41/43 of the dim CD20 cases. L26 staining is not usually graded by pathologists. The original report by Mason *et al.*, recommended that hematopathologists report all lymphomas that react with antibody L26 as "CD20 - positive" regardless of intensity[8]. However, the intensity of CD20 staining by IHC in most of our "dim" cases could not be distinguished from our "bright" cases. The dynamic resolution of IHC is too low to detect this difference. Other alternative explanations for the discordance between FCM and IHC could be a conformational change in the extracellular domain prohibiting proper binding of the B1 antibody to its epitope. For example, interleukin-4 may induce a conformational change in CD20 to prevent one but not other antibodies from binding to their extracellular epitopes[32]. Importantly, we have shown that mutations in exon 5 of the *MS4A1* gene that codes for the extracellular domain of the CD20 protein do not explain discrepancy between FCM and IHC. PCR-

based direct sequencing would not, however, detect complete loss of one copy of the gene, nor would it detect loss of exon 5. Methylation of the promoter of the gene causing a decrease in its transcription would also not be detected by this technique.

Discordant CD20 is not synonymous with “dim” or negative FMC7. Negative or “dim” FMC7 was more common and unlike discordant CD20, was not predictive of overall survival. In a study by Hübl *et al.* investigating CD20 and FMC7 intensity in various lymphomas, 2/11 (19%) of their “aggressive” lymphoma samples (mainly DLBCL) were FMC7-negative and CD20-positive which is in agreement with our results[33]. In addition, they and others found that the correlation between CD20 and FMC7 is the lowest in CLL and that little additional information is gained by using FMC7 if the intensity of CD20 expression is considered[33,34]. Interestingly, Polyak *et al.* found that FMC7 may be an indicator of membrane cholesterol content as cholesterol depletion markedly diminishes the expression of FMC7[17,35]. Thus samples with discordant CD20 may represent a form of DLBCL that has an altered membrane cholesterol metabolism.

Reduced levels of membrane CD20 could be associated with other confounding factors that were not measured in this study. In CLL, CD20 expression was recently shown to be associated with specific cytogenetic alterations and clinical outcome[36]. For instance, trisomy 12 was associated with a high CD20 expression and the best response to rituximab whereas 11q deletions were

associated with the lowest CD20 expression and the worst responses to rituximab[36]. Although we cannot exclude 11q deletions as a potential cause of discordant CD20, these genetic events are too infrequent in DLBCL to be the sole explanation for the relatively high incidence of discordant CD20 observed in our study[37]. Interestingly, the majority of our discordant CD20 and CD5+ samples had positive staining for BCL2 protein suggesting that inhibition of apoptosis may be involved in these cases. These results are in agreement with the recent study looking at the outcome of CD5+ DLBCL where 90% of CD5+ biopsies were also BCL2 protein positive[21]. Although CD5+ is associated with clinical outcome, our Cox regression analysis suggests that discordant CD20 or high-risk clinical features, not CD5+, is the main contributor of the negative prognostic effect of CD5+ DLBCL. Another possibility is that discordant CD20 may be surrogate marker for cells that are "frozen" at a different stage of differentiation reflected by a slightly higher proportion of ABC subtypes in the discordant CD20 group.

Discordant CD20 appears to be a marker for a more aggressive DLBCL biology given its association with poor survival in CHOP only treated patients. The B cell receptor (BCR) is crucial to B cell survival and signalling and it is modulated by co-receptors such as CD19, CD20 and CD5 (see reviews by Feske *et al.* and Monroe *et al.*)[38-41]. CD19 and CD20 both function as calcium (Ca^{2+}) channels, and through their interaction with the BCR, direct B cell fate through various pathways including activating NFκB[41]. These receptors aggregate together on

lipid rafts that act to compartmentalize and stabilize BCR signaling[42,43]. Recently, it was shown that lymphoma cells are dependent on Ca^{2+} entry into the cell in order to be killed by rituximab and that the Ca^{2+} influx by CD20 is dependant on BCR[44,45]. Finally, as with FMC7, reduced surface CD20 may reflect an imbalance in cholesterol and lipid metabolism in the tumour cells. For instance, the levels of ganglioside GM1 by FCM, used frequently as a marker for lipid rafts, has recently been shown to be highly correlated with rituximab response in cell lines and primary lymphoma samples[46]. Cross linking of CD20 antigen by rituximab onto lipid rafts appears to be important in mediating rituximab induced apoptosis and complement dependent cytotoxicity. Thus *in vitro* evidence confirms an important role for CD20 and CD19 in lymphoma biology.

The results of this study appear to identify a group of high-risk patients that may be good candidates for novel targeted therapies. Indeed, 13% of patients with DLBCL had discordant CD20 on their diagnostic biopsies and the majority (63%) developed a lymphoma relapse after R-CHOP. The high proportion of discordant CD20 cases with strong BCL2 protein expression suggests that these tumours may be "BCL2 dependent" and may benefit from targeted therapy with novel BH3 mimetics that bind to and inhibit BCL2 family proteins[47]. Another approach may be to use newer generations of anti-CD20 monoclonal antibodies that may be more active in lymphomas with a low CD20 density. These fully humanized antibodies appear to be more effective than rituximab at mobilizing CD20 onto lipid rafts and activating complement dependent cytotoxicity. These agents have

already been shown to be active and safe in phase I/II clinical trials that have included patients with relapsed CLL[48-50]. Thus identifying patients with discordant CD20 at the time of diagnosis could be crucial as they may derive the most benefit from these novel agents. Furthermore, FCM is considered routine in many clinical laboratories and thus the recognition of these patients is already possible with currently available data.

In conclusion, we have demonstrated that discordant CD20 expression by FCM using diagnostic DLBCL biopsies may be a novel biomarker that could identify a subgroup of high risk patients treated with R-CHOP. Moreover, this biomarker could be identified using flow cytometry, a technique that is already used in most clinical laboratories. More sensitive methods of quantifying CD19 and CD20 expression should be studied further to determine their association with outcome in different lymphoma subtypes. Studies to explore the basis of the inter-patient heterogeneity in expression, for example by assessing the methylation status of the gene, are also warranted. Currently, CD20-positive staining by IHC, not FCM, is one of the criteria for inclusion into clinical trials investigating the activity of novel anti-CD20 agents. Determination of CD20 and CD19 expression by FCM may be very helpful in these patients because it would allow more efficient investigation of novel anti-CD20 agents that may be able to overcome the negative prognostic effect of CD20 discordance. If so, we may reduce lymphoma relapses due to discordant CD20 by identifying high-risk patients early and treating them with more effective first line therapy.

Clinical characteristics	CHOP treated n = 82 (%)	R-CHOP treated n = 190 (%)
Age > 60 years old	41 (50)	107 (56)
Male sex	51 (63)	128 (67)
PS > 1	30 (37)	66 (35)
LDH > normal	46 (56)	90 (47)
Extranodal sites > 1	14 (17)	39 (21)
Stage III/IV	45 (55)	97 (51)
IPI score at diagnosis:		
0	12 (15)	26 (14)
1-2	32 (40)	91 (48)
3-4-5	37 (45)	73 (38)
Pathology of biopsy:		
DLBCL	82 (100)	181 (95)
PMBCL		9 (5)
Site:		
nodal	70 (85)	145 (76)
extranodal	12 (15)	45 (24)
Relapse or progression	38 (46)	55 (29)

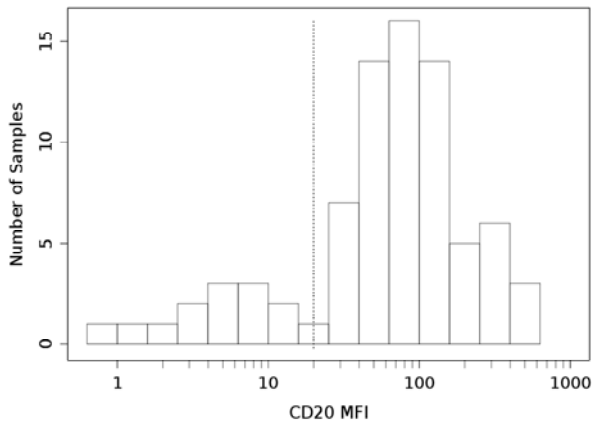
Table 3.1: Patient characteristics
Abbreviations: PS: ECOG performance status; LDH, lactate dehydrogenase; IPI, international Prognostic Index; DLBCL, diffuse large B cell lymphoma; PMBCL, primary mediastinal B cell .lymphoma; IHC, immunohistochemistry;

Variables	Discordant CD20 N = 35 (%)	CD5 expression N = 24 (%)
Age>60 years old	22 (63)	14 (58)
Male sex	26 (74)	16 (64)
PS > 1	16 (45)	11 (46)
LDH > normal	24 (69)	15 (62)
Extranodal sites > 1	10 (29)	6 (25)
Stage III/IV	32 (91)	14 (60)
IPI score at diagnosis:		
0	3 (9)	2 (8)
1-2	9 (26)	9 (38)
3-4-5	23 (65)	13 (54)
CD5 expression	11 (31)	24 (100)
Discordant CD20 by FCM	35 (100)	13 (54)
Bright CD20 expression by IHC	33 (94)	24 (100)
BCL2 protein expression	28 (81)	20 (83)
DLBCL subtype (Cell of origin):		
GCB	6 (17)	5 (21)
Non-GCB, ABC or Unclassifiable	12 (34)	8 (33)
Not available	17 (49)	11 (46)
Relapse or progression	22 (63)	12 (50)

Table 3.2: Clinical and pathological characteristics of patients with DLBCL biopsies having discordant CD20 or CD5 expression
Abbreviations**:** PS: Eastern Cooperative Oncology Group (ECOG) performance status; LDH, lactate dehydrogenase; IPI, international Prognostic Index; IHC, immunohistochemistry; “Discordant CD20”, reduced CD20 but bright CD19 expression by flow cytometry

	Bright CD20 expression	Dim CD20 expression
Bright CD19 expression	203 (75 %)	**35 (13 %)**
Dim CD19 expression	26 (10 %)	8 (3 %)

Table 3.3: Incidence of DLBCL samples stratified according to CD19 and CD20 expression



Figures 3.1 A: Distribution of mean fluorescence intensity (MFI) in CD20 expression in the DLBCL samples from 2004-2007

Abbreviations: MFI, mean fluorescence intensity (PE-Cy5); DLBCL, diffuse large B cell lymphoma

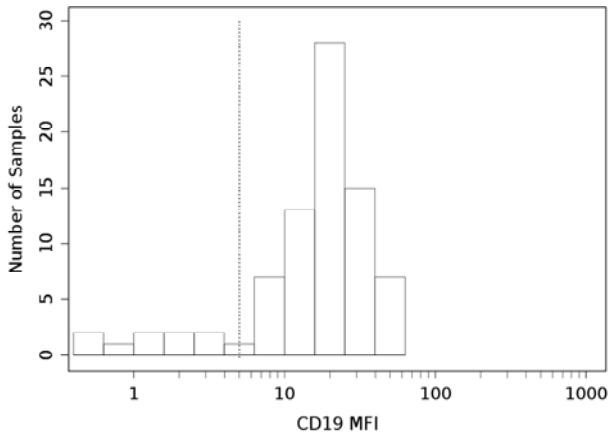


Figure 3.1 B: Distribution of mean fluorescence intensity (MFI) in CD19 expression in the DLBCL samples from 2004-2007

Abbreviations: MFI, mean fluorescence intensity (PE-Cy5); DLBCL, diffuse large B cell lymphoma

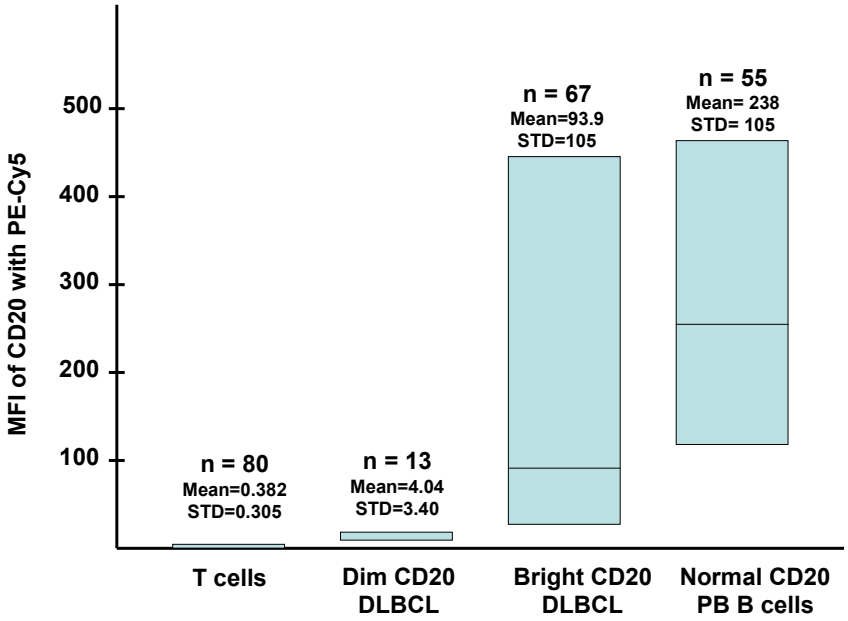


Figure 3.1 C: Heterogeneity in CD20 expression in DLBCL and normal peripheral blood lymphocytes (2004-2007)

Abbreviations: MFI, mean fluorescence intensity; DLBCL, diffuse large B cell lymphoma; STD, standard deviation; PB, peripheral blood;
From 2002-2004: dim CD20 mean MFI: 2.1, STD: 0.8, range: 0.55-3.91; bright CD20 mean MFI: 17.6, STD: 19.4, range: 4.13-103
From 1997-2002: dim CD20 mean MFI: 1.1, STD: 0.4, range: 0.69-1.92; bright CD20 mean MFI: 14.1, STD: 18.5, range: 2.4-85.1

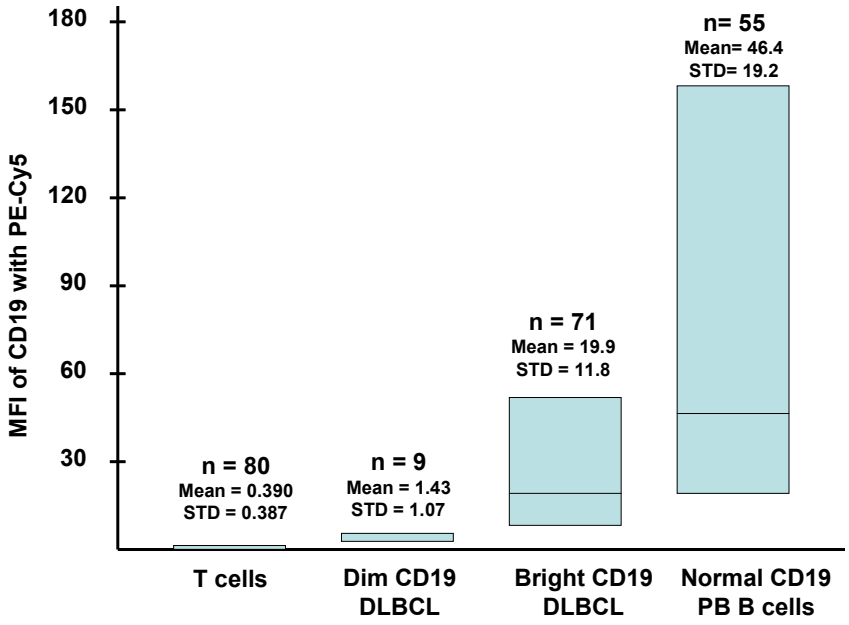


Figure 3.1 D: Heterogeneity in CD19 expression in DLBCL and normal peripheral blood lymphocytes (2004-2007)

Abbreviations: MFI, mean fluorescence intensity; DLBCL, diffuse large B cell lymphoma; STD, standard deviation; PB, peripheral blood;
From 2002-2004: dim CD19 mean MFI: 0.44, STD: 0.11, range: 0.27-0.57; bright CD19 mean MFI: 4.79, STD: 4.28, range: 0.6-17.5
From 1997-2002: dim CD19 mean MFI: 0.52, STD: 0.22, range: 0.15-0.85; bright CD19 mean MFI: 6.71, STD: 6.70, range: 1.79-36.5

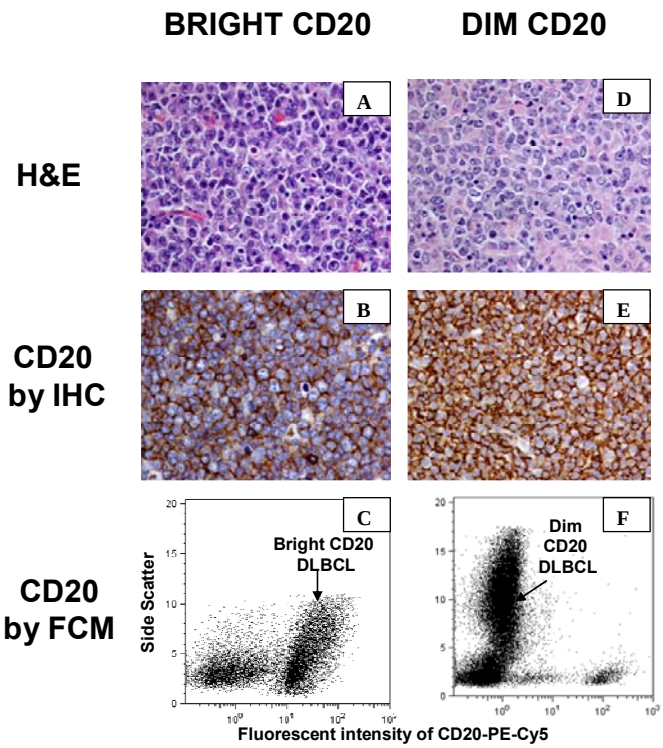
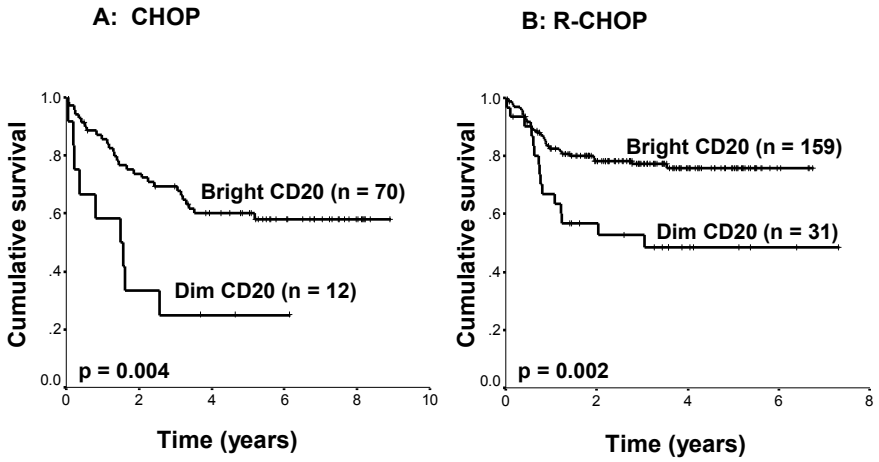


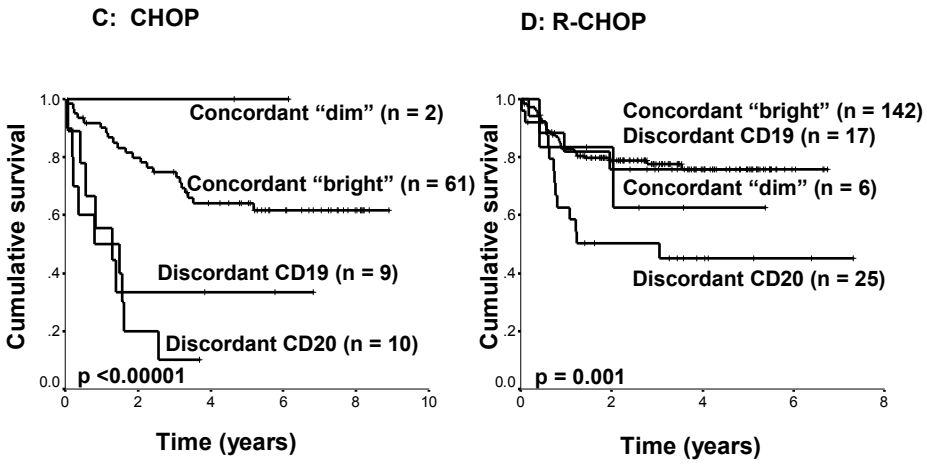
Figure 3.2: CD20 expression by immunohistochemistry and flow cytometry of representative "dim" CD20 and "bright" CD20 DLBCL samples

a) Representative hematoxylin and eosin (H&E) stain of a "bright" CD20 DLBCL
b) CD20 protein expression by immunohistochemistry of a "bright" CD20 DLBCL
c) CD20 expression by flow cytometry of a "bright" CD20 DLBCL
d) Representative hematoxylin and eosin (H&E) stain of a "dim" CD20 DLBCL
e) CD20 protein expression by immunohistochemistry of a "dim" CD20 DLBCL
f) CD20 expression by flow cytometry of a "dim" CD20 DLBCL



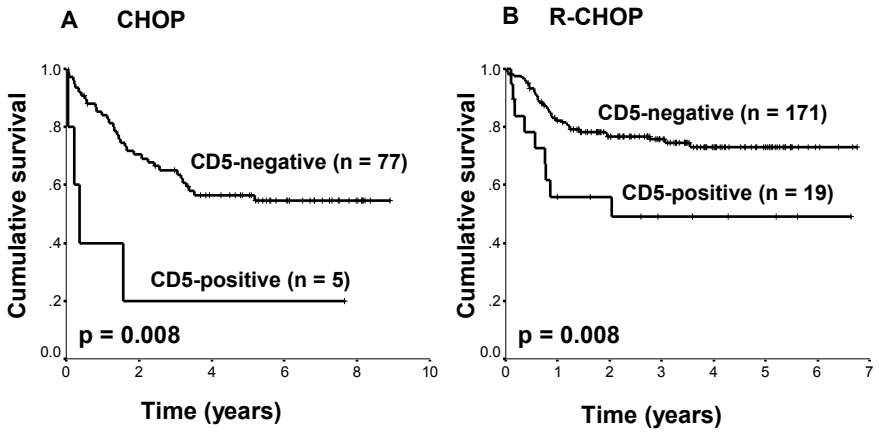
Figures 3.3 A and B: Overall survival of patients with DLBCL according to CD20 expression

a) CHOP treated
b) R-CHOP treated



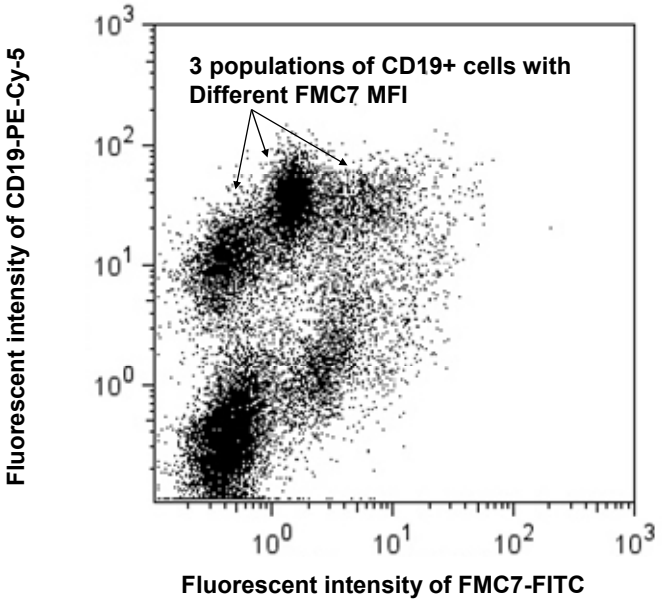
Figures 3.3 C and D: Overall survival of patients with DLBCL according to CD20 and CD19 expression

c) CHOP treated
d) R-CHOP treated



Figures 3.4 A and B: Overall survival of patients with DLBCL according to CD5 expression

a) CHOP treated
b) R-CHOP treated



Supplemental Figure 3.1: Three different populations of CD19+ cells with different FMC7 (CD20 antigen) fluorescence intensities

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Chapter 4: Lymphomas with concurrent *BCL2* and *MYC* translocations: the critical factors associated with survival*

*A version of this chapter has been published. Johnson N.A. et al. (2009) Lymphomas with concurrent *BCL2* and *MYC* translocations: the critical factors associated with survival. Blood.114:2273-9.

4.1 Introduction

BCL2 and *MYC* are two dominant acting oncogenes that are often de-regulated as a result of chromosomal translocation in B cell lymphomas. The translocation t(14;18) juxtaposes *BCL2* on chromosome band 18q21 to the immunoglobulin heavy chain gene (*IGH*) enhancer at band 14q32 resulting in BCL2 protein over-expression and inhibition of apoptosis[1-3]. It is found in 85% of follicular lymphomas (FL) and 15-30% of *de novo* diffuse large B-cell lymphomas (DLBCL)[1]. The translocation t(8;14)(q24;q32) is characterized by rapid cellular proliferation as a result of *MYC* de-regulation driven by the *IGH* enhancer[2,3]. This translocation is the hallmark of classical Burkitt lymphomas (BL)[2] but it is also found in a high proportion of cases previously known as atypical Burkitt/Burkitt like lymphoma (BLL) (41-80%)[4,5] and a subset of DLBCL (~5-8%)[6,7]. BLL are often associated with complex cytogenetic alterations and have an unfavorable clinical outcome compared to DLBCL and BL[4,5]. In the 2008 World Health Organization (WHO 2008) criteria for the classification of lymphomas, the term BLL has been dropped in favour of B cell lymphoma, unclassifiable, with features intermediate between BL and DLBCL[8]. In this study, this lymphoma category will be referred to as B cell lymphoma, unclassifiable (BCLU)[9].

Infrequently, *BCL2* and *MYC* translocations may be found concurrently in the same specimen, so-called “double hit disease”[8]. These cases may present with variable morphologies including acute lymphoblastic leukemia/lymphoma

(ALL), DLBCL, BCLU and rarely FL[10-16]. The primary event in these cases is usually the t(14;18) with the *MYC* translocation (*MYC*$^+$) arising as a secondary genetic event[10,17]. Patients with lymphomas harbouring concurrent *BCL2* and *MYC* translocations, hereafter referred to as *BCL2*$^+$/*MYC*$^+$ lymphomas, have been described in a number of small case series as having a very poor overall survival (OS)[13,14]. A recent study at our institution revealed that 4% (6/142) of patients with primary DLBCL had dual translocations involving both *BCL2* and *MYC* [18]. The clinical outcome of these patients was variable.

We conducted a comprehensive investigation of a large cohort of lymphomas with concurrent translocations of 8q24 (*MYC*) and 18q21 (*BCL2*) to identify the clinical and cytogenetic prognostic factors associated with survival.

4.2 Methods

Patient identification

Patients with non-Hodgkin lymphoma (NHL) diagnosed between 1991 and 2007 were identified in the British Columbia Cancer Agency (BCCA) Lymphoid Cancer and Cytogenetic Databases. We initially selected patients based on three requirements: accurate histological diagnosis re-assigned according to the recently published 2008 WHO classification for lymphoid neoplasms, availability of cytogenetic analysis by karyotype and detailed information on clinical outcome[9]. Indications for karyotype analysis included high grade histology, DLBCL with a high proliferation index and FL with atypical morphological

features. A total of 1118 patients met these criteria. In addition, fluorescence *in-situ* hybridization (FISH) analysis using commercial probes for *MYC* and *BCL2* (see cytogenetic section) was performed on a tissue micro-array (TMA) constructed from duplicate 0.6 mm cores of formalin-fixed paraffin embedded tissue (FFPET) derived from 142 unselected diagnostic samples of DLBCL[18]. Thus information on *MYC* and *BCL2* translocation status was available on 1260 patients. Of these, 54 patients were identified as having concurrent translocations at 18q21 and 8q24 (49 by karyotype and 5 by FISH).

Cases were considered to have BCLU if their biopsy revealed lymphoma with features intermediate between DLBCL and BL (one of the so-called grey zone lymphomas), previously called BLL. Standard diagnostic criteria were used to identify DLBCL and FL. Baseline clinical characteristics including the International Prognostic Index (IPI) variables were recorded[19]. Overall survival (OS) was calculated from the date of the *MYC*$^+$ biopsy to the date of last follow up or death from any cause. Progression free survival (PFS) was calculated from the date of the *MYC*$^+$ biopsy to the date of last follow up or disease progression. The chemotherapy regimens utilized for treatment of these patients were heterogeneous and based on treatment era. CHOP-like regimens included cyclophosphamide, doxorubicin, vincristine and prednisone. Rituximab was added to CHOP (R-CHOP) for aggressive histology B-cell lymphomas at our institution in March 2001 and this regimen was used in 11 patients (5 BCLU and 6 DLBCL)[20]. Palliative regimens included observation, radiation, immunotherapy

alone and non-anthracycline based chemotherapy. High dose chemotherapy included ALL type regimens (n = 2)[21] and high dose chemo-radiotherapy followed by hematopoietic stem cell transplant (autologous n = 3 and allogeneic n = 1). Ethical approval to perform this retrospective study was granted by the University of British Columbia - British Columbia Cancer Agency Research Ethics Board and is in accordance with the Declaration of Helsinki (REB# H08-02834).

Immunohistochemistry

Immunohistochemical analysis (IHC) of archived FFPET included CD20 (L26, Dako, CA), CD3 (Dako, CA), CD10 (Clone 56C6, Vector, CA), Ki-67 (Dako, CA) and BCL2 (clone 124, Dako, CA). Five cases had no BCL2 protein expression using clone 124 (targeting amino acids 41–54) and were subsequently analyzed using clone E17 (Epitomics, CA) (targeting amino acids 60 to 80). BCL2 protein or Ki-67 staining were not performed on bone marrow biopsies or peripheral blood. Cases were considered positive for CD10 and BCL2 if >30% of the neoplastic cells stained positively. The proliferation rate was determined in 20 samples using the monoclonal antibody Ki-67 which binds to a nuclear protein in cycling cells. A cutoff value of ≥ 80% was used to define a high proliferation index according to the Southwest Oncology Group study by Miller *et al*.[22].

Genomic sequencing of the BCL2 gene

The five cases that were BCL2 protein-negative by clone 124 were investigated for mutations in the *BCL2* gene. Genomic DNA was extracted using the ALL

PREP kit (QIAGEN, Germany) or the PureGene DNA purification kit (GENTRA, MN) in three frozen samples and two FFPET samples, respectively. The *BCL2* genomic sequence corresponding to amino acids 8 to 126 of the BCL2 protein was PCR amplified using the following primers containing the universal -21M13F and M13R sequencing tags (italics):
Forward 5'-*TGTAAAACGACGGCCAGT*GGGTACGATAACCGGGAGAT-3' and Reverse 5'-*CAGGAAACAGCTATGAC*CGGCGGGAGAAGTCGT-3'. The purified 334 base pair product was bi-directionally sequenced using BigDye® Terminator v3.1 (Applied Biosystems) and an ABI 3730 XL sequencer (Applied Biosystems). Mutations were considered present if they were observed in both forward and reverse reads.

Cytogenetic Analysis

G-banded karyotype and multicolor karyotype (MFISH) analyses were performed as previously reported[23,24]. Karyotype descriptions conform to the International System for Human Cytogenetic Nomenclature (ISCN) 2005[25]. *MYC* rearrangements were confirmed using the LSI *MYC* dual color break-apart probe (Abbott Molecular, Abbott Park, IL) on cells fixed in methanol: acetic acid (3:1) in 48/54 cases (including the 5 cases identified by TMA). Additional BAC probes were used to further characterize the *MYC* breakpoints and partner chromosomes involved in the *MYC* translocations (see Supplemental Table 4.1). The BAC probes were directly labeled by nick translation using a commercial labeling kit according to the manufacturer's protocol (Abbott Molecular, Abbott

Park, IL). Forty samples, including the five samples that were BCL2 protein-negative were confirmed to have t(14;18) involving the *BCL2* gene by FISH using the LSI *IGH/BCL2* dual color, dual fusion translocation probe (Abbott Molecular, Abbott Park, IL). For probe signal scoring, a minimum of 200 interphase nuclei were examined. A cutoff threshold of > 5% positive cells was used to confirm the presence of *IGH-BCL2* and *IGH-MYC* translocations. The cell line Karpas 353, which contains the translocation t(8;9), was utilized as control material for the FISH experiments, generously provided by Dr. A. Karpas[26].

Statistical Analysis

Statistical analyses were performed using SPSS software version 11.0 and the R statistical package (http://cran.r-project.org/). Fisher's exact test and likelihood ratios were used to determine the significance of any differences between patients with different histological and cytogenetic characteristics. Survival curves were plotted using the Kaplan Meier method and compared using the log-rank test. Hazard ratios and 95% confidence intervals were calculated using univariate Cox proportional-hazards models. In the multivariate model, we included terms that appeared to be important on univariate analyses and then used a backward selection method to remove the non-significant terms from the model. A $p < 0.05$ (two sided) was considered statistically significant.

4.3 Results

A total of 1260 cases of NHL were identified in the BCCA lymphoid cancer and cytogenetics databases between 1991 and 2007 as having the required clinical information, pathology review and cytogenetic analysis available for this study. Of these, 54 cases (4%) were identified as having concurrent translocations involving *MYC* and *BCL2* based on karyotype and/or FISH analysis. These samples were acquired from patients having different diagnoses and treatment regimens over a 16 year period.

Clinical characteristics

MYC rearrangements were found at the time of initial lymphoma diagnosis in 31 cases (57%). In 23 cases (43%) the *MYC* rearrangement occurred at the time of histological transformation from a pre-existing FL (n = 19) or chronic lymphocytic leukemia (CLL) (n = 1). Three patients had an antecedent DLBCL. Extranodal disease was common and 59% had bone marrow (BM) and/or peripheral blood (PB) involvement at the time of detection of the *MYC* rearrangement. Treatments for the prior indolent lymphomas had included observation (n = 6), radiation (n = 4), single or multi-agent chemotherapy with or without immunotherapy (n = 9) or this information was not available (n = 1). Treatment for the $BCL2^{+}/MYC^{+}$ lymphomas consisted of CHOP (n = 23), R-CHOP (n = 11), high dose chemotherapy +/- SCT (n = 6) and palliation (n = 14).

Immunohistochemical and Immunophenotypic characteristics

The *BCL2*$^+$/*MYC*$^+$ lymphomas frequently presented as a BCLU (n = 36) or DLBCL (n = 17). One sample had a *MYC*$^+$ lymphoma with FL morphology (grade 2). A high proliferation index, as determined by a Ki-67 ≥ 80%, was observed in 14/20 of tested cases (including two of the five BCL2 clone 124 - negative cases) and tended to correlate with a BCLU morphology, but in this limited number of samples, this observation was not statistically significant (p = 0.15). Immunophenotyping analysis revealed that 62% of samples co-expressed CD19, CD20 and CD10 consistent with a germinal center phenotype.

Genetic Analysis of the *BCL2* gene

Despite the presence of t(14;18), five *BCL2*$^+$/*MYC*$^+$ lymphomas were considered to be BCL2 protein-negative by clone 124 that is used routinely in most clinical laboratories. Four of these were considered BCL2 protein-positive with the E17 antibody. Synonymous and non-synonymous mutations in the *BCL2* gene were detected in two of the three interpretable cases. These two cases had discrepant staining with both antibodies and harboured mutations in the flexible loop domain but not the BH3 domain (see Figure 4.1). Both mutated cases had C →T mutations that are typical of somatic hypermutation as previously described by Tanaka *et al*[27]. No mutations were detected in the one sample that was negative by both antibodies. However, mutations 5' of the amplified sequence or in the promoter regions of the *BCL2* gene could not be excluded. The patient with the "true" BCL2-negative biopsy is alive, free of disease >6 years after the *MYC*$^+$

diagnosis. Two of the FFPET cases had poor quality sequence data and mutations could not be excluded or confirmed.

Cytogenetic characteristics

By karyotype, 30/54 cases had *MYC* translocations involving the *IG* loci (16 t(8;14), 11 t(8;22) and 3 t(2;8)). Of the cases with t(8;14), eight had a complex rearrangement [t(14;18)t(8;14)] where *MYC* was adjacent to the 3'*IGH* enhancer on derivative chromosome 14 and *BCL2* was driven by the 5'*IGH* enhancer relocated to the derivative chromosome 8 as previously described[28]. The remaining *MYC* rearrangements involved variant chromosome partners, most commonly a t(8;9)(q24;p13) (13/24). The other *MYC* partner loci included 1p36, 3p25, 3q27*(BCL6)*, 4p13, 5q13, 12p11 and 13q31. Multiple alterations were present in almost all cases in addition to the *BCL2* and *MYC* translocations including breakpoints at chromosome band 3q27 (13%) and loss of 17p13 (16%) or 1p36 (36%). These results confirm previous observations that $BCL2^{+}/MYC^{+}$ lymphomas, unlike classic BL, typically have complex karyotypes[29].

Given that t(8;9) was the most frequent non-*IG/MYC* rearrangement observed , a series of adjacent BACs were used to determine the breakpoint site at 9p13 in the cell line Karpas 353 and in 11/13 of the clinical cases (see Supplemental Figure 1). The breakpoint region covered by BACs RP11-220I1 through RP11-405L18 spans ~400 kb of genomic region just 5' of the *PAX5* locus as previously described by Bertrand *et al*[30].

Characteristics associated with overall survival

Table 4.1 demonstrates the effect of each clinical, immunophenotypic and cytogenetic factor on OS for the entire cohort. Every death in this study, except for one, was attributed to lymphoma progression thus PFS was very similar to OS (data not shown). As illustrated in Figure 4.2a, patients identified as having a *MYC*$^{+}$ rearrangement at the time of their initial lymphoma diagnosis had a similar outcome to those who acquired it at the time of transformation. Only 6/54 patients remained alive and in remission over a median follow-up time of 5.3 years, three of which presented at diagnosis and three at relapse. Thus samples in these two groups were pooled together in subsequent analyses.

Figure 2b demonstrates that 32 (59%) patients died within six months following the diagnosis of the *MYC*$^{+}$ rearrangement irrespective of treatment regimen. The addition of rituximab to CHOP may have improved outcome in patients with *BCL2*$^{+}$*/MYC*$^{+}$ lymphomas (median OS 1.4 years vs. 0.4 years for R-CHOP (n=11) and CHOP (n=23) treated patients, respectively ($p = 0.05$)), however, too few patients were treated with R-CHOP to support any firm conclusions. The six patients treated with high dose chemotherapy and SCT, all of which had BCLU histology, had a similar poor outcome compared to those patients treated with palliation (median survival 3 month vs. 1 month, $p > 0.05$) suggesting that even intensified therapy cannot overcome the aggressive tumour biology in these patients. Of the ten patients with OS >2 years, the predominant histology was

DLBCL (9/10) and their treatment regimens included CHOP (n = 6) and R-CHOP (n = 4).

Restricting the analysis to the 40 patients treated with curative intent, which excludes the case of FL, five factors were associated with a more favourable outcome: a low IPI score, the absence of bone marrow involvement, DLBCL histology, the presence of a non-*IG/MYC* partner and a BCL2 protein-negative biopsy (clone 124) (Figure 4.3). There was a strong association between DLBCL morphology and the presence of a non-*IG/MYC* partner and a BCL2 protein-negative biopsy ($p < 0.0001$ and $p = 0.04$, respectively). Conversely, BCLU morphology was associated with the presence of an *IG/MYC* partner and a bone marrow/leukemic presentation ($p = 0.001$). Of the 4 variables presented in Figure 4.3, only bone marrow/leukemic presentation of MYC^+ lymphoma was associated with a high IPI score ($p=0.03$). Other factors not associated with survival were a high proliferation index, CD10 expression or the presence of additional cytogenetic alterations such as a rearrangement at 3q27, a loss of 17p13 or 1p36.

Given the small sample size, multivariate Cox regression analysis was performed excluding BCL2 as a variable because this result was not available in most patients. Hazard ratios for the univariate and multivariate models are listed in Table 4.3. If only three variables are entered into the model, then morphology

and IPI, not the *MYC* translocation partner, were the most predictive factors of OS (morphology: p = 0.0001; IPI 2-3 vs. 0-1: p=0.290, IPI 4-5 vs. 0-1: p=0.0086).

4.4 Discussion

This study demonstrates that lymphomas with concurrent *BCL2* and *MYC* translocations are heterogeneous in morphology, clinical presentation and outcome. The non-descript term "double hit" lymphomas does not adequately capture the full clinical spectrum of this disease. It is more precise and clinically relevant to refer to these lymphoma subtypes using the appropriate morphologic classification, DLBCL versus B cell lymphoma, unclassifiable, with features intermediate between BL and DLBCL, with an indication of the dominant oncogenes involved, including the *MYC* translocation partner and the BCL2 protein expression.

BCL2^{+}*/MYC*$^{+}$ lymphomas may be under-recognized because this diagnosis would be missed if genetic testing by karyotype and/or FISH analysis was not performed. These lymphomas represented 4% of selected lymphoma cases subjected to karyotype and FISH analysis in British Columbia. A selection bias in our study may have lead to a modest over-estimation of the incidence given that cytogenetic analysis was selectively performed on lymphoma samples that had high grade histology or aggressive clinical features. However, the incidence of *MYC* translocations may also have been under-estimated because some non-*IG*/*MYC* rearrangements could have been missed by karyotype analysis alone.

Furthermore, FISH analysis could miss uncommon *MYC* breakpoints that are centromeric or telomeric to the commercial break-apart probes used in most clinical labs[31,32]. Indeed, one of our cases had a t(8;14) by karyotype and a far centromeric *MYC* breakpoint that was not detected using the commercial LSY *MYC* Dual Color break apart probe [31]. Therefore, more sensitive *MYC* FISH probes may be needed to eliminate the false negative results with the current commercial probes. With those limitations in mind, it is interesting that our estimate is comparable to that seen in a comprehensive review of 2175 lymphoma karyotypes[33]. A 5% incidence of *MYC* translocations was reported amongst 355 cases of t(14;18) positive lymphomas (2% had t(8;14), 2% had t(8;22) and 1% had t(8;9)) [33]. Furthermore, the incidence of dual *MYC* and *BCL2* translocations in *de novo* DLBCL was found to be 3-4% in two independent studies[18,34].

The IPI and tumour morphology appear to be the most powerful, independent, predictors of outcome in this disease, the latter being highly associated with the *MYC* translocation partner. *MYC* is a crucial regulator of all aspects of cellular growth and proliferation (reviewed by Wierstra *et al.*)[35] In the case of the t(8;14) and related *IG/MYC* translocations, *MYC* is under the regulation of a strong *IG* enhancer resulting in constitutive *MYC* expression that commonly manifests as a higher tumour grade. The gene expression profiling study by Hummel *et al*. showed a strong correlation between the *MYC* partner, *MYC* expression and resultant morphology[36]. In our study, non-*IG/MYC* translocations were common

and the “classic” t(8;14) translocation was present in only 15% of our samples. The chromosomal band 9p13 was the most common non-*IG MYC* translocation partner and the breakpoints flanked several candidate genes including *ZCCHC7* and were approximately 200 kb 5’ of *PAX5* [30]. These genes are transcription factors that are very important in B cell development and could potentially lead to *MYC* deregulation when translocated near the 8q24 locus [37,38]. Within both the DLBCL and BCLU categories, there was a trend towards a more favorable outcome for patients having non-*IG*/*MYC* translocation partners in their biopsies thus FISH analysis using only a *MYC* break-apart probe may not be sufficient for accurate prognostication. Future prospective studies to investigate the clinical impact of *MYC* translocation partners in larger cohorts of DLBCL and BCLU are required.

Mutations in the *BCL2* gene corresponding to the flexible loop domain may be clinically important and may explain the observed discrepancies between the results with the different BCL2 antibodies (clone 124 and E17). Such discrepancies have been observed in FL and are thought to be a consequence of ongoing somatic hypermutation given the proximity of *BCL2* to the *IGH* locus[27,39]. Such mutations may interfere with BCL2 function sufficiently to affect the caspase cleavage site at D34, the P53 binding site at amino acids 32 to 68 or the phosphorylation of S70 which is required for BCL2’s full and potent anti-apoptotic function[40,41]. Thus, it is conceivable that the BCL2 “pseudo-negative” cases in our study may have a dysfunctional BCL2 protein that is associated with

improved chemo-sensitivity to R-CHOP. Rituximab has been shown to partially overcome the chemotherapy resistance associated with BCL2 protein expression (positive by clone 124) in DLBCL[42]. Stolz *et al.* have recently shown that rituximab can induce apoptosis through the "mitochondrial" pathway that is regulated by the BCL2 protein family and the addition of BH3 mimetics that target BCL2 function can restore chemo-sensitivity in primary rituximab resistant cell lines [43]. Indeed, the addition of the BH3 mimetic ABT-737 to cyclophosphamide was synergistic at inducing long term remissions in 78% (14/18) of mice with $BCL2^+/MYC^+$ lymphomas[44]. Thus pharmacologic modulation of BCL2 in humans with BH3 mimetics +/- rituximab may render the aggressive $BCL2^+/MYC^+$ lymphomas chemo-sensitive and should be studied further in the context of clinical trials.

In summary, a comprehensive cytogenetic analysis of *BCL2* and *MYC* status is currently feasible in most reference clinical laboratories and should be performed on all aggressive lymphomas as this may not only rule out BL as a potential diagnosis, but it may identify more homogeneous populations of lymphomas within the current BCLU category[29]. Concurrent *BCL2* and variant (non-*IG*) *MYC* rearrangements may be more common than previously appreciated in DLBCL. Prospective analysis of their incidence and prognostic significance, including BCL2 protein expression, in patients with DLBCL treated with R-CHOP may lead to the identification of very high-risk patients that might derive the most benefit from rituximab and BCL2 targeted therapy. Furthermore, it may pave the way to

a better understanding of the complex synergism that arises when these two dominant oncogenes are deregulated.

Variables	N = 54 (%)	Median OS (years)	Log rank P value
Age > 60 years at diagnosis	28 (52)	0.58	0.0028
Male gender	32 (59)	0.32	0.8252
Performance status >1	19 (35)	0.27	0.0010
Stage >2	41 (76)	0.30	0.0647
LDH> normal	27 (50)	0.35	0.9234
Extranodal sites > 1	19 (35)	0.18	0.0005
IPI			
0-1	16 (30)	1.35	0.0001
2-3	24 (44)	0.48	
4-5	14 (26)	0.15	
Age at *MYC* rearrangement > 60 years	32 (59)	0.26	0.0046
Antecedent lymphoma without *MYC*		0.41	0.5301
FL	19 (35)		
CLL	1 (2)		
DLBCL	3 (6)		
Histology with *MYC* rearrangement			<0.0001
DLBCL	17 (31)	2.86	
BCLU	36 (67)	0.26	
FL	1 (2)		
Bone Marrow involvement by *MYC*			0.0006
Positive	32 (59)	0.20	
Negative	13 (24)	1.40	
Not Available	9 (17)		
BCL2 protein expression			0.0095
Positive	23 (43)	0.48	
Negative	5 (9)	5.66	
Not available	27 (48)		
CD10 expression			0.3162
Positive	34 (63)	0.30	
Negative	9 (17)	0.20	
Not available	11 (20)		
IG/MYC rearrangement	30 (56)	0.26	0.0170
Non-*IG/MYC* rearrangement	24 (44)	0.58	
Treatment:			
HD chemo	6 (11)	0.26	0.0005
R-CHOP	11 (20)	1.40	
CHOP-like	23 (43)	0.42	
Palliative	14 (26)	0.07	

Table 4.1: Characteristics associated with overall survival in all 54 patients with BCL2+/MYC+ lymphomas Abbreviations: LDH, lactate dehydrogenase; IPI, international Prognostic Index; FL, follicular lymphoma; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B cell lymphoma; BCLU, B cell lymphoma, unclassifiable, with features intermediate between Burkitt lymphoma and DLBCL; *IG*, immunoglobulin; HD chemo, high dose chemotherapy with or without stem cell transplant; R-CHOP, rituximab and CHOP chemotherapy

	Univariate			Multivariate		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
BCLU vs. DLBCL	5.73	2.49 - 13.2	**<0.001**	4.04	1.40 - 11.6	0.009
BM^+ vs. BM^-	3.27	1.50 - 7.33	**0.004**	1.98	0.79 - 5.03	0.150
$BCL2^+$ vs. $BCL2^-$	2.86	0.791 – 10.3	0.110			
IG/*MYC* vs. non-IG/*MYC*	3.42	1.62 - 7.22	**0.001**	1.47	0.55 - 3.88	0.441
IPI Risk Group 2-3 vs. 0-1	1.64	0.747 – 3.58	0.220	1.45	0.53 – 3.95	0.470
IPI Risk Group 4-5 vs. 0-1	5.12	1.84 – 14.31	**0.002**	3.25	0.85 – 12.4	0.084

Table 4.2: Univariate and multivariate models using overall survival for 40 patients with concurrent *BCL2* and *MYC* translocations treated with curative intent

Abbreviations: HR, hazard ratio; CI, confidence interval; DLBCL, diffuse large B cell lymphoma; BCLU, B cell lymphoma unclassifiable, with features intermediate between Burkitt lymphoma and DLBCL; BM, bone marrow involvement; BCL2, BCL2 protein expression (clone 124); *IG*, immunoglobulin; IPI, international Prognostic Index

A

Case	Dako clone 124 Amino acid sequence 41-54	IHC	Epitomics E17 Amino acid sequence 60-80	IHC
Ref	AAPAPGIFSSQPGH	P	ASRDPVARTSPLQTPAAPGAA	P
1	AA**S**APGIFSSQPGH	N	ASRDPVARTSPLQTPAAPGAA	P
2	AAPA**SV**IFSSQPGH	N	ASRDPVARTSPLQTPAAPGAA	P
3	AAPAPGIFSSQPGH	N	ASRDPVARTSPLQTPAAPGAA	N

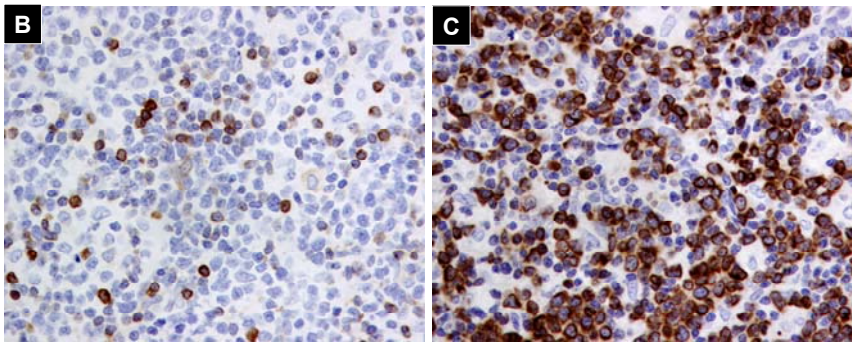


Figure 4.1: Correlation between the presence of mutations in the *BCL2* gene and BCL2 protein expression by immunohistochemistry using clones 124 and E17

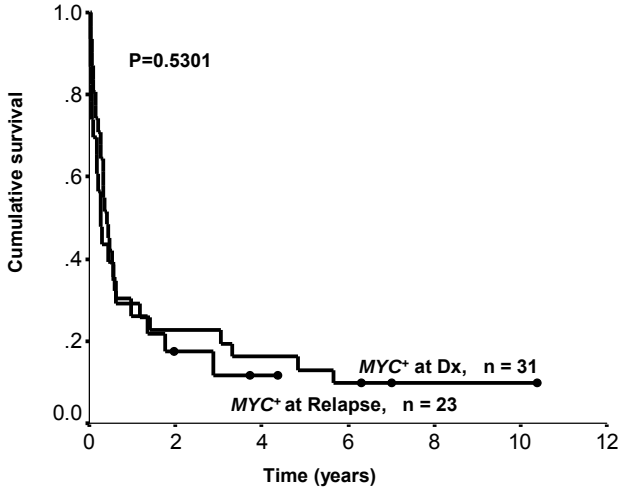
A. BCL2 protein expression by immunohistochemistry and corresponding amino acid sequence derived from sequencing the *BCL2* gene in three *BCL2*$^{+}$/*MYC*$^{+}$ lymphomas samples. The affected amino acid changes are highlighted in red.

B. 200 x magnification of a DLBCL sample (sample 2) stained with the BCL2 antibody clone 124 (Dako)

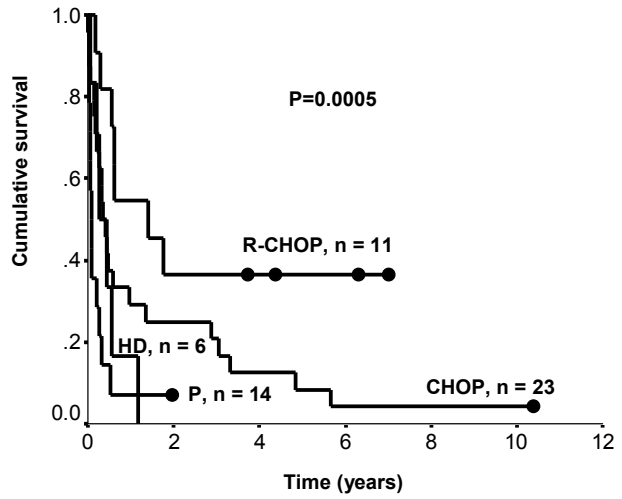
C. 200 x magnification of the same DLBCL sample (sample 2) stained with the BCL2 antibody E17 (Epitomics)

Abbreviations: Ref, reference genome hg 19, May 2009; IHC, immunohistochemistry; P, positive; N, negative

A



B



Figures 4.2 A and B: Survival curves of patients with *BCL2*$^{+}$/*MYC*$^{+}$ lymphomas according to the timing of *MYC*$^{+}$ rearrangement (A) and treatment regimen (B)

Abbreviations: Dx, diagnosis; R, rituximab; HD, high dose chemotherapy +/- stem cell transplant; P, palliative. Black circles indicate long term survivors.

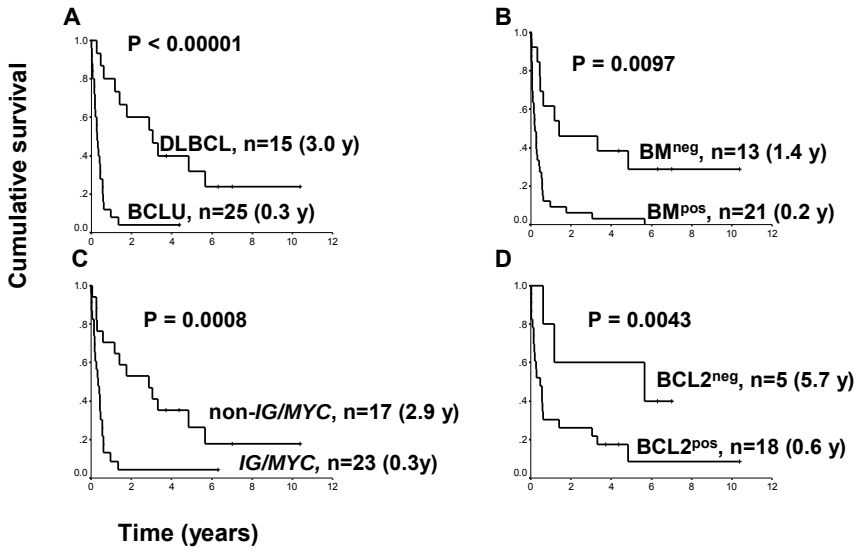


Figure 4.3: Survival curves of patients with *BCL2*$^{+}$/*MYC*$^{+}$ lymphomas according to morphology, bone marrow involvement, *MYC* translocation partner and BCL2 protein expression

Median overall survivals are shown in parentheses.

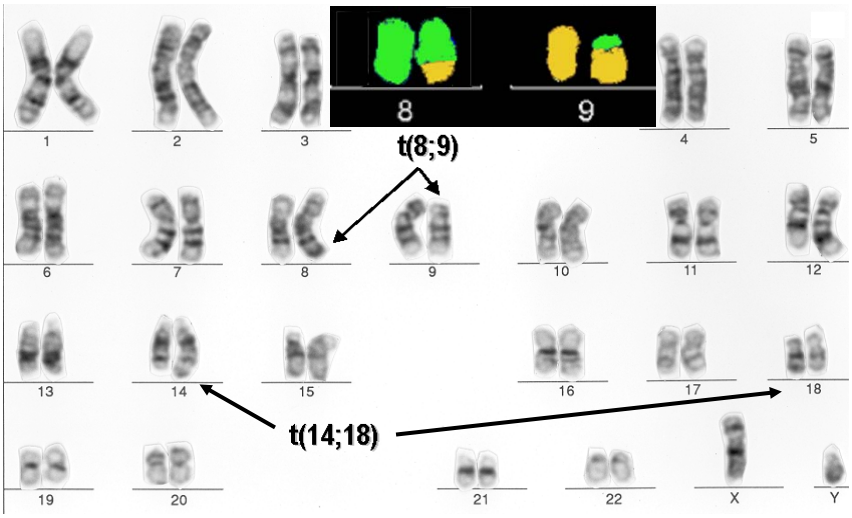
A. Morphology

B. Bone marrow involvement

C. *MYC* translocation partner

D. BCL2 protein expression (clone 124)

Abbreviations: BCLU, B cell lymphoma unclassifiable; DLBCL, diffuse large B cell lymphoma; BM^{neg}, no bone marrow involvement with *MYC*$^{+}$ lymphoma; BM^{pos}, bone marrow involvement with *MYC*$^{+}$ lymphoma; BCL2^{neg}, no BCL2 protein expression by clone 124; BCL2^{pos}, BCL2 protein expression by clone 124; *IG/MYC*, *MYC* translocation involving one of the immunoglobulin genes.



Supplemental Figure 4.1: Chromosome breakpoint analysis in lymphomas with translocation t(8;9) and t(14;18)

Representative karyotype: translocation t(8;9) and t(14;18)

Gene	Breakpoint	Centromeric BAC	GAP	Telomeric BAC
MYC	8q24.1	RP11-440N18 RP11-367L7 RP11-495D4* RP11-781C3*		RP11-125A17 RP11-748F3 RP11-440N18* RP11-125A17*
		319kb 378kb*	87kb 1.7Mb*	331kb 439kb*
PAX5	9p13.1	RP11-663O12 RP11-263I4 RP11-644E22		RP11-117L21 RP11-344B23
		655kb	347kb	460kb
BCL2	18q21.3	RP11-53O13		RP11-165H6
		160kb	396kb	184kb
IGL	22q11.2	RP11-22M5 RP11-114D2 RP11-359L2 RP11-69B15 RP11-761L13		RP11-1087B15 RP11-124F9 RP11-76E8
		395kb	883kb	611kb
IGK	2p11.2	RP11-31G9 RP11-97F19 RP11-136K15		RP11-554H10 RP11-645N19 RP11-39F20 RP11-1023A24
		342kb	839kb	349kb

Supplemental Table 4.1: List of BACs used to determine the MYC translocation partners in addition to the commercial probes

*far centromeric breakpoint *MYC* as per Hummel M, Bentink S, Berger H, et al. A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. N Engl J Med. 2006;354:2419-2430 (Supplementary Appendix).

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Chapter 5: BCL2 protein expression determines clinical outcome in MYC-positive DLBCL patients treated with R-CHOP*

A version of this chapter will be submitted for publication. Johnson N.A. et al. (2010). BCL2 protein expression determines clinical outcome in MYC-positive DLBCL patients treated with R-CHOP.

5.1 Introduction

The distinction between Burkitt lymphoma (BL) and diffuse large B cell lymphoma (DLBCL) is clinically important because patients with these types of cancer are managed differently, the latter being treated rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone (R-CHOP) and the former, more intensive regimens [1-4]. The hallmark of BL is the presence of a translocation involving the oncogene c-*MYC* (8q24) with one of the immunoglobulin genes (*IG*) on chromosomes 14 (*IGH*), 2 (*IGκ*), or 22 (*IGλ*), that results in *MYC* over-expression and rapid cellular proliferation[5-7]. *MYC* translocations are not specific to BL and can also be detected in ~ 7-10% of diffuse large B cell lymphoma (DLBCL) and in 40-80% of B cell lymphoma unclassifiable with features intermediate between DLBCL and BL, hereafter referred to as BCLU[8-10]. BL karyotypes have been described as "*MYC* simple", whereby the *MYC* oncogene is translocated to an IG gene and few (<2), if any, additional cytogenetic alterations are present. In contrast, DLBCL and BCLU usually have more complex karyotypes (*MYC* complex) and over-express BCL2 protein, an oncogene that inhibits apoptosis[8,11].

In 2006, two groups performed gene expression profiling (GEP) on classic BL and DLBCL cases to determine a gene expression signature that could accurately classify these two diseases at the molecular level, hoping to also improve on the ability to diagnose challenging borderline cases[12,13]. However, there remained a subset of cases with morphological features of DLBCL but a molecular BL signature at the mRNA level. These discrepant cases harbored

MYC translocations and 50% of them had a concurrent *BCL2* translocation[12]. The presence of concurrent *MYC* and *BCL2* translocations is generally associated with a poor overall survival (OS) but certain features have been associated with a more favourable outcome, specifically those associated with a DLBCL morphology, *MYC* translocations involving a non-*IG* gene translocation partner and tumour cells that fail to express BCL2 protein[14]. However, studies to date have included a heterogeneous group of patients treated mainly in the pre-rituximab era. Investigating the role of BCL2 in the context of a *MYC* translocation is important because the addition of rituximab to CHOP was shown to overcome the negative prognostic impact of BCL2 protein expression in DLBCL[15].

Recently, high *MYC* mRNA expression and the presence of a *MYC* translocation have both been independently reported to be associated with a poor OS in DLBCL treated with R-CHOP raising controversy regarding the optimal management of these high-risk patients[16,17]. We determined whether the prognostic effect of high *MYC* expression or a *MYC* translocation in DLBCL is influenced by the concurrent presence of a *BCL2* translocation or BCL2 protein expression. Furthermore, we also assessed the clinical impact of gene expression signatures including the molecular BL signature[12].

5.2 Methods

Patient identification

Pre-treatment tumour biopsies taken from 180 patients with *de novo* DLBCL obtained from 10 international institutions were assembled for this study. A panel of expert hematopathologists confirmed a consensus diagnosis of DLBCL using the World Health Organization criteria of 2008[1]. Primary mediastinal large B cell lymphoma, BL and BCLU were excluded from this analysis. The patients were treated with curative intent with R-CHOP and their initial clinical characteristics, including the International Prognostic Index (IPI) and clinical outcomes, were recorded. A total of 158/180 (88%) were included in the previous report by Lenz *et al.* and 49/180 (27%) were included in the analysis by Savage *et al.*[17,18]. Ethical approval to perform this study was granted by each institution's Research Ethics Board in accordance with the Declaration of Helsinki.

Specimen processing and immunohistochemistry

All biopsies had sufficient tumour material such that both formalin-fixed paraffin embedded tissue (FFPET) and fresh frozen tissue were available for the study. Tissue microarrays (TMA) were constructed at each institution using duplicate or triplicate 0.6 mm cores of FFPET. Presence of BCL2 protein expression was determined by immunohistochemistry (IHC) using the clone 124 (Dako, CA) and was considered positive if > 30% of the tumour cells stained for the antibody.

Fluorescent in situ hybridization (FISH)

The presence of translocations involving *BCL2, MYC* and *BCL6* was determined using commercial dual color "break-apart" probes from Abbott Molecular (Abbott Park, IL) according to the manufacturer's protocol using FFPET as previously described[17]. In *MYC* translocated cases, break-apart probes for *IGH*, *IGκ* and *IGλ* (Dako, CA) were further used to determine the *MYC* translocation partner (*IG* versus non-*IG).* Given that the BCL6 gene is also translocated to IG genes in DLBCL, the BCL6 probe was mainly used to help classify cases as IG versus non IG. Cases with break-apart signals (individual red and green) in >5% cells were called "positive" for a translocation. Three cases were deemed indeterminate because they had concurrent *MYC*, *BCL2* and *BCL6* translocations (n=2) or the *IGH* result was un-interpretable (n=1).

Gene expression profiling

RNA was extracted from frozen tissue using the ALL PREP kit (QIAGEN, Germany) and was reverse-transcribed and hybridized to Affymetrix HG U133 Plus 2.0 arrays (Affymetrix, CA) as previously published by Lenz *et al*[18]. CEL files were normalized using robust multi-chip analysis (RMA) and samples were assigned a molecular subtype (molecular BL, germinal center B-cell type (GCB), activated B cell type (ABC) and unclassifiable (U) according to the expression of classifier genes in the study by Dave *et al*[12,19]. Hierarchical clustering of samples was performed using R on a subset of molecular BL signature genes that were selected based on their association with a molecular BL subtype

(molecular BL versus DLBCL) using global test, z-score >10[20]. *MYC* expression was determined using log normalized expression values of probe set id 202431_s_at and dichotomized into high versus low expression using a cut off threshold determined by the statistical software X-Tile (>9.4 = high) (http://www.tissuearray.org/rimmlab/). A moderated t-test (limma package) was used to select genes that were significantly different ($p < 0.05$) between high *MYC* (n=5) and low *MYC* (n=40) expressing cases that were also of the ABC subtype and BCL2 protein-positive[21]. Adjusted p values were calculated according to the Benjamini-Hochberg (BH) method to control for the false discovery rate due to multiple testing[22]. Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, www.ingenuity.com) was used to determine the biological pathways associated with the 100 most significant genes from this analysis.

Statistical Analysis

Progression free survival (PFS; event = progression or death from any cause after the start of chemotherapy) and overall survival (OS; event = death from any cause) were estimated using the Kaplan Meier method and differences were assessed using the log rank test. The Cox Proportional Hazards model included factors that had p values of <0.05 in univariate analysis. The Pearson Chi-Square test was used to compare variables between the different molecular subtypes and an independent sample t test was used to compare *MYC* expression levels in cases based on the presence of *MYC* translocations. The above statistical analyses were performed using SPSS software version 11.0.

5.3 Results

A total of 180 DLBCL tissue samples had available clinical information, good quality GEP arrays and FFPET on TMAs. Of these, 170/180 had technically successful FISH results for *MYC* translocation status and were included in the final analysis. Their baseline clinical characteristics are shown in Table 5.1. With a median follow-up of 3.5 years for living patients, the median OS for the entire 170 patients was > 7 years.

Molecular Burkitt signature is rare in DLBCL and not associated with poor survival

The molecular BL signature was only present in 2 of the 170 DLBCL cases (< 1%). Their clinical presentation was similar to classic BL in that both patients were male and had bulky abdominal masses with involvement of the gastrointestinal tract as an extra-nodal site. Both biopsies were BCL2 protein-negative and by definition had very high *MYC* expression. One of the two cases had a detectable *MYC* translocation (*IG/MYC*). Unexpectedly, although both patients were over 60 years old, each had an excellent response to R-CHOP and had maintained a complete remission for more than two years.

ABC DLBCL has a poorer outcome than GCB DLBCL

Patients with the ABC molecular subtype (n = 70) had an inferior outcome with a median survival of 5.6 years *versus* not reached for GCB, U and molecular BL subtypes (p=0.003), independent of IPI in multivariate analysis. Furthermore, the

ABC subtype was associated with high-risk clinical features such as higher IPI scores and more frequent expression of high levels of BCL2 protein and/or *MYC* mRNA (all $p < 0.01$).

MYC translocation-positive cases have heterogeneous *MYC* expression

MYC translocations (18/170, 10%) were detected in all molecular subtypes (GCB=10/74(13%), ABC =6/70 (9%), U=1/24, molecular BL=1/2, see Table 5.1) and were associated with higher *MYC* expression compared to non-translocated cases (9.22 vs 8.22, $p = 0.0001$, see Table 5.2). *IG/MYC* translocations occurred in 9/18 (50%) cases and correlated with higher *MYC* RNA expression although this did not reach statistical significance (mean *IG/MYC* RNA expression= 9.66 vs non-*IG/MYC* RNA expression = 8.81, $p=0.2$). Hierarchical clustering of samples according to their expression of molecular BL classifier genes demonstrated that *IG/MYC* translocation cases tended to cluster closer to molecular BL cases while non-*IG MYC* translocation cases were more heterogeneous (see Figure 5.1). *BCL2* and *BCL6* translocations were present in 29 (17%) and 34 (20%) cases. *BCL2* translocations were predominantly detected in the GCB subtype (23/74 (31%), $p=0.005$) whereas *BCL6* translocations were associated with the ABC subtype (20/70 (29%), $p=0.018$). Concurrent *MYC* and *BCL2* translocations occurred in 5/170 (3%) patients. Of these, two cases also had evidence of additional *BCL6* translocations (so-called “triple-hit” disease). In summary, among the 170 DLBCL cases, 18 (10%) had detectable *MYC*

translocations but only 9 of these showed concomitant over-expression of *MYC* mRNA.

MYC translocations are associated with an inferior survival only when BCL2 protein is expressed

The presence of a *MYC* translocation was associated with an inferior PFS (p=0.038) and there was a trend towards an inferior OS (p=0.057). However, the presence of concurrent *MYC* and *BCL2* translocations (n=5) was associated with a markedly inferior OS compared to either *MYC* translocation alone (no *BCL2* translocation) or no *MYC* translocation (median OS of 6 months versus not reached and not reached, respectively, p<0.0001). Of the 18 patients with *MYC* translocations, 11 also had over-expression of BCL2 protein. These *MYC* translocated-BCL2-protein positive cases had a median OS of 11 months compared to median OS not reached in other cases (p= 0.0007, Figure 5.2a). The presence of either a *BCL2* or *BCL6* translocation alone was not associated with clinical outcome in the entire group of 170 patients or within the GCB or ABC molecular subtypes (data not shown).

High *MYC* expression is associated with a poor survival in association with BCL2 protein expression

High *MYC* RNA expression was detected in 19/170 (11%) biopsies and was associated with an inferior OS, p=0.02. However, patients whose biopsies showed high expression levels of *MYC* RNA and BCL2 protein (n=10) had a significantly inferior median OS (11 months compared to not reached for the

other cases, p=0.0004, Figure 5.2b). Interestingly, 5 of these 10 patients had evidence of a *MYC* translocation while the other 5 had an ABC molecular subtype without a *MYC* translocation. We considered whether the latter five cases were biologically different than other ABC DLBCL samples. These five patients with high *MYC* expressing ABC DLBCL appeared to have an inferior OS compared to the other 40 ABC DLBCL patients with low *MYC* expression, BCL2 protein-positive lymphoma (median OS of 1.38 y versus 6 years, p=0.013). Only eight genes showing a BH adjusted p value of <0.05 were significantly differentially expressed between these two groups, of which *MYC* was the top gene (BH p=0.0005)[22]. Using IPA software and examining the top 100 differentially expressed genes ranked according to adjusted p value, we determined that the distinguishing pathways between high vs low *MYC* RNA ABC cases were cell cycle progression and cellular proliferation with *E2F3*, *TFDP1* and *MYC* being the three genes that were most significantly over-expressed in the high *MYC* RNA group (see Supplemental figure 5.1).

"High risk" versus "low risk" *MYC* deregulation DLBCL is determined by BCL2 protein expression

In total, 28 cases had either high *MYC* expression or a *MYC* translocation (see Figure 5.3). Deregulation of both the *BCL2* and *MYC* oncogenes occurred in 16/170 (9 %) of *de novo* DLBCL biopsies and was associated with inferior OS and PFS following R-CHOP (median OS and PFS of <1 year versus not reached, p<0.0001, see Figure 5.2c). These 16 cases included the 11 that harboured

MYC translocations and were also BCL2 protein-positive (including the 5 cases with dual *BCL2* and *MYC* translocations) and 5 additional cases that had high *MYC* expression (but without *MYC* translocation) and were BCL2 protein-positive. These 16 "high risk" patients presented with a poor performance status (8/16, p=0.004), higher LDH levels (6/16, p=0.054) and consequently higher IPI scores (p=0.03) compared to patients whose tumours had no evidence of a *MYC* alteration (n=142) or patients in the "low risk MYC" group (n=12). In a Cox-multivariate model, the IPI, cell of origin and "high risk *MYC"* were all independent predictors of OS and PFS (see Table 5.3 for hazard ratios).

The most common mechanism of high *MYC* expression was a *MYC* translocation (9/19), which can be detected by standard cytogenetic or FISH analysis. We determined if *MYC* expression correlated with mRNA expression levels of the *KI67* gene given that Ki-67 protein expression correlates with cellular proliferation by IHC. If positively correlated, one could potentially use this as a surrogate marker for high *MYC* expression in cases without evidence of a *MYC* translocation. The highest correlation between *MYC* and *KI67* expression was r = 0.2 for probe set 212022_s_at, suggesting that Ki-67 protein might not be a useful surrogate marker for *MYC* expression.

5.4 Discussion

In this study, we have demonstrated that *BCL2*, a key regulator of apoptosis, has a crucial role in determining cell fate when *MYC*, the master regulator of the cell

cycle, is de-regulated in DLBCL. This study improves our understanding of DLBCL biology and has important clinical implications both from a diagnostic and treatment standpoint in this disease.

DLBCL cases with *MYC* translocations likely represent a spectrum of biology. Herein we have shown that BCL2 protein expression clearly impacts behavior of the neoplastic B cells. It is likely that the underlying genetic complexity may also distinguish subsets within the *MYC* translocated cases, analogous to *MYC*-simple versus *MYC*-complex as previously described by Boerma and colleagues[11]. It is reasonable to hypothesize that *MYC*-simple cases maybe those where the translocation of the *MYC* oncogene is a primary genetic event and represents the cases with a molecular BL gene expression signature. In contrast, the *MYC*-complex cases may be heterogeneous, with at least some cases where the *MYC* translocation is a secondary genetic event and the gene expression pattern is therefore not characteristic of molecular BL.

Despite genomic heterogeneity, DLBCL can have homogeneous phenotypes, i.e. cases where oncogenes cooperate to promote cell cycle progression and inhibit apoptosis. Both *MYC* and *BCL2* expression can be de-regulated through a variety of mechanisms including alteration of the gene locus (e.g. translocations, amplifications, mutations, etc) or by targeting upstream signaling pathways such as *NOTCH1* and *NFκB*[5,23-26]. The heterogeneity in *MYC* expression observed in this study can in part be explained by the *MYC* translocation partner with different

enhancer/promoter elements. *MYC* orchestrates the expression of ~15% of the genes in the genome and functions as a major control hub downstream of several cellular pathways[27]. In addition to promoting cell cycle progression, *MYC* can induce apoptosis by directly increasing P53 expression or amplifying the signaling in the intrinsic and extrinsic apoptotic pathways (reviewed by Hoffman and Lievermann) [28-30]. Thus, it appears that despite deregulation of *MYC*, cell fate may ultimately reside on the ability of the cell to undergo apoptosis, suggesting the latter may be a potential target for therapy in this subset of cases.

Our results suggest that R-CHOP can be effective for patients with "low risk *MYC*" DLBCL, i.e. *MYC* is deregulated but BCL2 protein is not expressed. However, DLBCL patients with "high risk *MYC*" (deregulated *MYC* and BCL2 protein-positive) DLBCL have a median OS <1 year after R-CHOP, thus more aggressive treatment regimens should be considered for these patients. These results are in keeping with the study by Savage *et al.*, as 9/12 cases with *MYC* translocations in that study were BCL2 protein-positive and although the BCL2 protein-negative cases had a favorable outcome similar to this study, no firm conclusions could be made at that time based on only 3 cases. In British Columbia, the rituximab-modified Magrath protocol used to treat BL, CODOX-M-IVAC +/- rituximab +/- autologous stem cell transplant, is under investigation for patients with lymphomas harboring concurrent *BCL2* and *MYC* translocations[4]. Interestingly, results from the recent MRC/NCRI LY10 trial which investigated the use of dose modified CODOX-M-IVAC, without rituximab, in patients with

biopsies with high proliferation rates (Ki-67 > 95%) showed that 4/5 cases with concurrent *BCL2* and *MYC* translocations died within 5 months of initiating therapy despite the use of high dose regimens[31].

More than 50% of the "high risk *MYC*" patients in this study had a poor performance status or older age that might preclude them from regimens more aggressive than R-CHOP. These confounding factors may in part explain the poor outcomes seen after standard regimens in patients with molecular BL or BCLU[12]. Thus additional cytogenetic investigations in patients not suitable for intensified treatment may only be justified in the context of clinical trials. The prominent role played by BCL2 in this disease suggests that these lymphomas may be very sensitive to BH3 mimetics, such as ABT-737 or ABT-263 (Navitoclax) [32,33]. Alternatively, type II anti-CD20 agents that can directly induce non-apoptotic cell death may overcome the negative prognostic effect of BCL2 over-expression and improve outcome in this disease[34]. Finally, histone deacetylase inhibitors and proteasome inhibitors can kill tumour cells expressing high levels of *MYC* by modulating the expression of the BCL2 protein family *in vitro*[35,36].

The identification of high-risk DLBCL patients who have *MYC* translocations and BCL2 protein-positivity would be possible if cytogenetic analysis using the commercial *MYC* break-apart FISH probe were performed on all DLBCL biopsies. Routine testing for both translocations would incur significant additional

costs to detect only 6% (11/170) of patients. A more cost effective approach may be to perform FISH studies in DLBCL samples that are BCL2 protein-positive. In this scenario, 11 cases with *MYC* translocations would be detected after testing only 93 biopsies (11%). Ideally, *MYC* mRNA expression should be investigated in clinical practice because it may be a surrogate marker for the activation of other oncogenes that increase cellular proliferation.

Determining the *MYC* and BCL2 status on all DLBCL patients whose lymphomas have relapsed following R-CHOP and who are enrolled in clinical trials investigating the use of novel biological agents would be valuable. These studies may lead to insights into optimal treatment strategies for patients not eligible for intensified therapy. Furthermore, it may provide a framework to build on when treating future patients who have other genomic alterations that also culminate in de-regulated cellular proliferation and apoptosis pathways.

Features	Total N = 170 (%)	mBL N = 2 (%)	GCB N = 74 (%)	ABC N = 70 (%)	U N = 24 (%)	p
IPI 0-1	83 (48)	0	44 (60)	23 (33)	16 (67)	0.007
2-3	67 (39)	1	22 (30)	38 (54)	6 (25)	
3-4	20 (12)	1	8 (11)	9 (13)	2 (8)	
BCL2 Protein +*	93 (55)	0	32 (43)	51 (71)	11 (46)	<0.001
FISH:						
***MYC*+**	18 (11)	1	10 (14)	6 (9)	1 (4)	0.184
IG	9	1	4	4	0	
Non-*IG*	6	0	4	1	1	
Indeterminate	3	0	2	1	0	
BCL2*+	29 (17)	0	23 (31)	4 (6)¥	2(8)	0.005
BCL6*+	34 (20)	0	8 (11)	20 (29)	5 (21)	0.018
BCL2+MYC+	5 (3)	0	2 (3)	2 (3)	1 (4)	
BCL6+MYC+	2 (1)	0	1 (1)	1 (1)	0 (0)	
BCL2+BCL6+MYC+	2 (1)	0	1 (1)	1 (1)	0 (0)	
Gene expression:						
↑*MYC* expression	19 (11)	2 (100)	5 (7)	10 (14)	2 (8)	<0.001

Table 5.1: Clinical characteristics of the 170 patients

Abbreviations: mBL, molecular Burkitt lymphoma; GCB, germinal center B cell lymphoma; ABC, activated B cell lymphoma; U, unclassifiable; IPI, international prognostic index; FISH, fluorescent in situ hybridization; +, presence of a translocation; *IG, MYC* translocation partner is one of the immunoglobulin genes; non-*IG, MYC* has a non-immunoglobulin gene translocation partner; Indeterminate, the assignment of translocation partner (*IG* vs non-*IG*) could not be determined.

* missing values in 3 cases for BCL2 protein, in 10 cases for *BCL2* translocation and 7 cases for *BCL6* translocation.

¥ = 2 of the cases had concurrent amplification of *BCL2* (>4 copies)

Features	Number of Cases	Mean *MYC* mRNA expression	Standard deviation	P value
Cell of origin				
mBL	2	10.97	0.16	<0.0001
GCB	74	8.00	1.01	0.001*
ABC	70	8.52	0.77	
Translocation				
***MYC*+**	18	9.22	1.23	<0.0001
***MYC*-**	126	8.22	0.87	
***BCL2*+/*MYC*+**	5	9.28	0.86	0.896**
IG/MYC	9	9.66	1.26	0.202
Non-*IG/MYC*	6	8.81	1.09	

Table 5.2: Correlation between *MYC* translocation status and *MYC* mRNA expression

Abbreviations: mBL, molecular Burkitt lymphoma; GCB, germinal center B cell lymphoma; ABC, activated B cell lymphoma; IG, immunoglobulin gene; +, presence of a translocation; -, absence of a translocation
*p value comparing GCB vs ABC

Variable	Incidence (%)	Univariate HR	P value	Multivariate HR	P value
IPI		18.26	0.0001	12.33	0.002
Cell of origin					
mBL vs GCB vs ABC vs U		14.04	0.0029	9.62	0.022
ABC vs non-ABC	70 (41)	13.66	0.0002		
MYC +	18 (11)	3.0	0.057		
BCL2 +/*MYC* +	5 (3)	40.43	<0.0001		
MYC + & BCL2 protein+	11 (7)	14.55	0.0007		
High *MYC* exp	19 (11)	5.34	0.0208		
High *MYC* exp & BCL2 protein+	10 (6)	15.52	0.0004		
"High risk *MYC*" ¥					
Deregulated *MYC*/BCL2 protein+	16 (9)	24.06	<0.0001	10.11	0.001

Table 5.3: Univariate and multivariate hazard ratios for variables associated with overall survival

Abbreviations: IPI, international prognostic index; mBL, molecular Burkitt lymphoma; GCB, germinal center B cell lymphoma; ABC, activated B cell lymphoma; U, unclassifiable; +, presence of a translocation; exp, mRNA expression

¥ High risk *MYC* includes 11 cases that MYC tr+ & BCL2 protein positive disease (including the 5 cases with dual BCL2 and MYC translocations) and 5 additional cases that had high MYC expression & BCL2 protein positive biopsies (all ABC subtype)

The p values in the multivariate analysis for progression free survival are: IPI, p=0.004; cell of origin, p=0.003; High risk *MYC*, p=0.05.

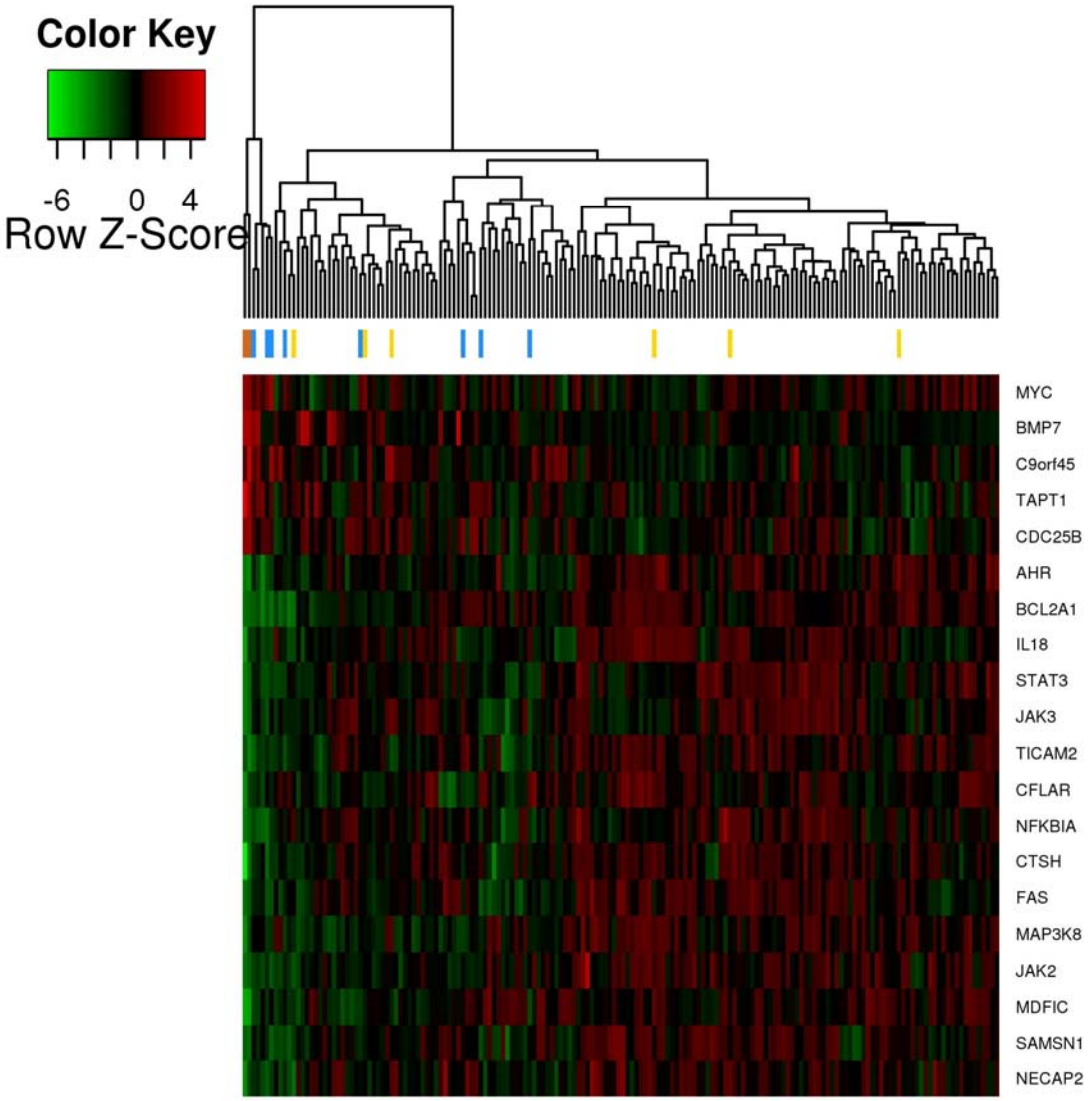


Figure 5.1: Hierarchical clustering according to *mBL* classifier genes

X axis: each column represents a different patient sample
Y axis: each row represents a different gene
The three cases that had *MYC* translocations but whose translocation partner could not be determined are not highlighted in this figure.

Legend: Vertical lines at the top of the heat map represent different molecular subtypes or *MYC* translocation partners. Red lines represent cases of molecular Burkitt lymphoma, blue and orange lines represent *MYC* translocated cases to immunoglobulin genes (IG) or non-IG partners respectively.

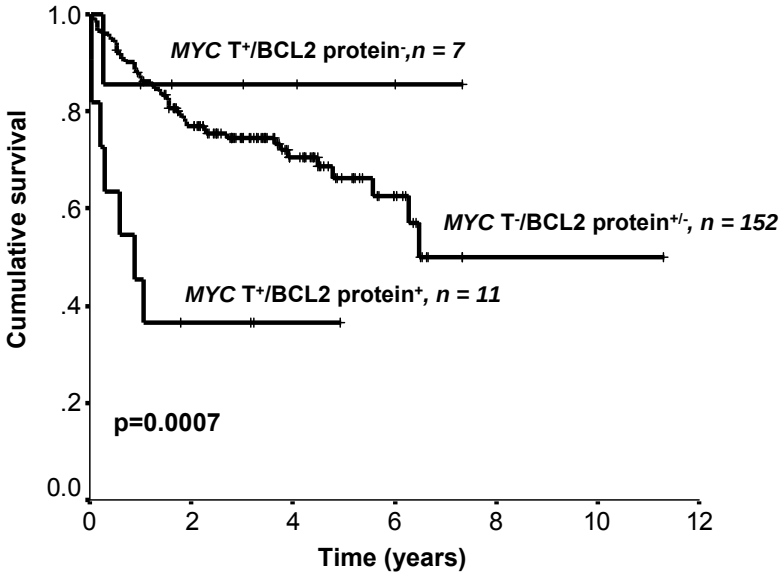


Figure 5.2 A: Overall survival according to the presence of *MYC* translocations and BCL2 protein expression

Abbreviations: T, translocation

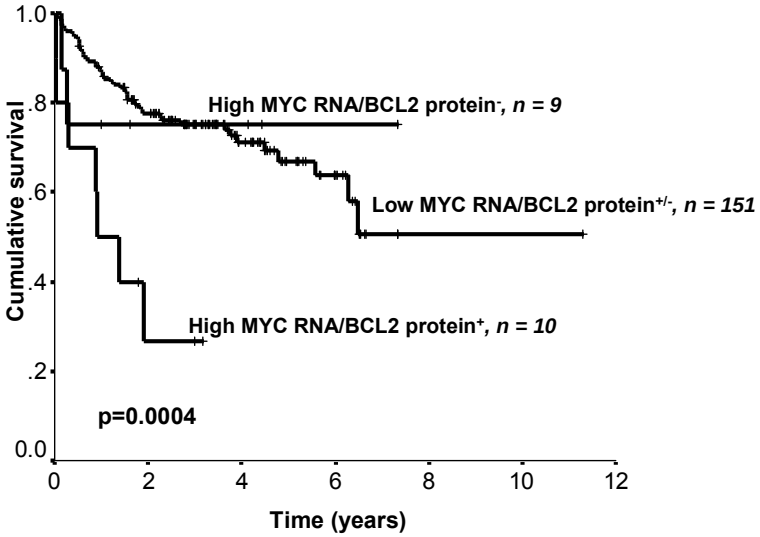


Figure 5.2 B: Overall survival according to the presence of high *MYC* expression and BCL2 protein expression

Abbreviations: exp, mRNA expression

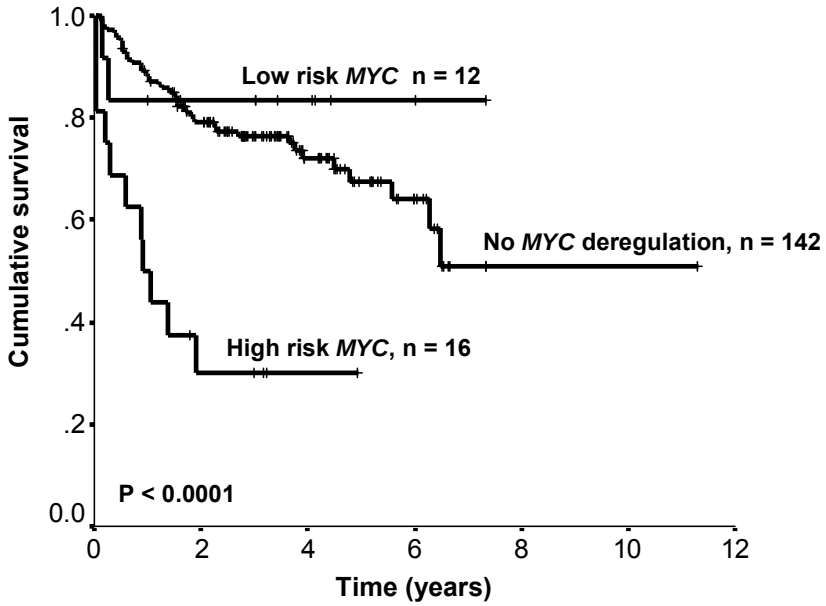


Figure 5.2 C: Overall survival according to *MYC* deregulation and BCL2 protein expression

Low risk: *MYC* deregulation and BCL2 protein-negative
High risk: *MYC* deregulation and BCL2 protein-positive

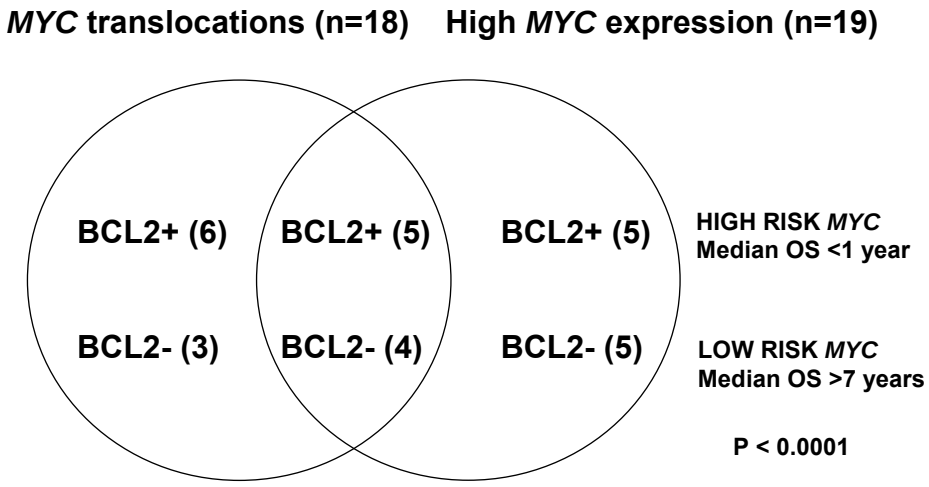
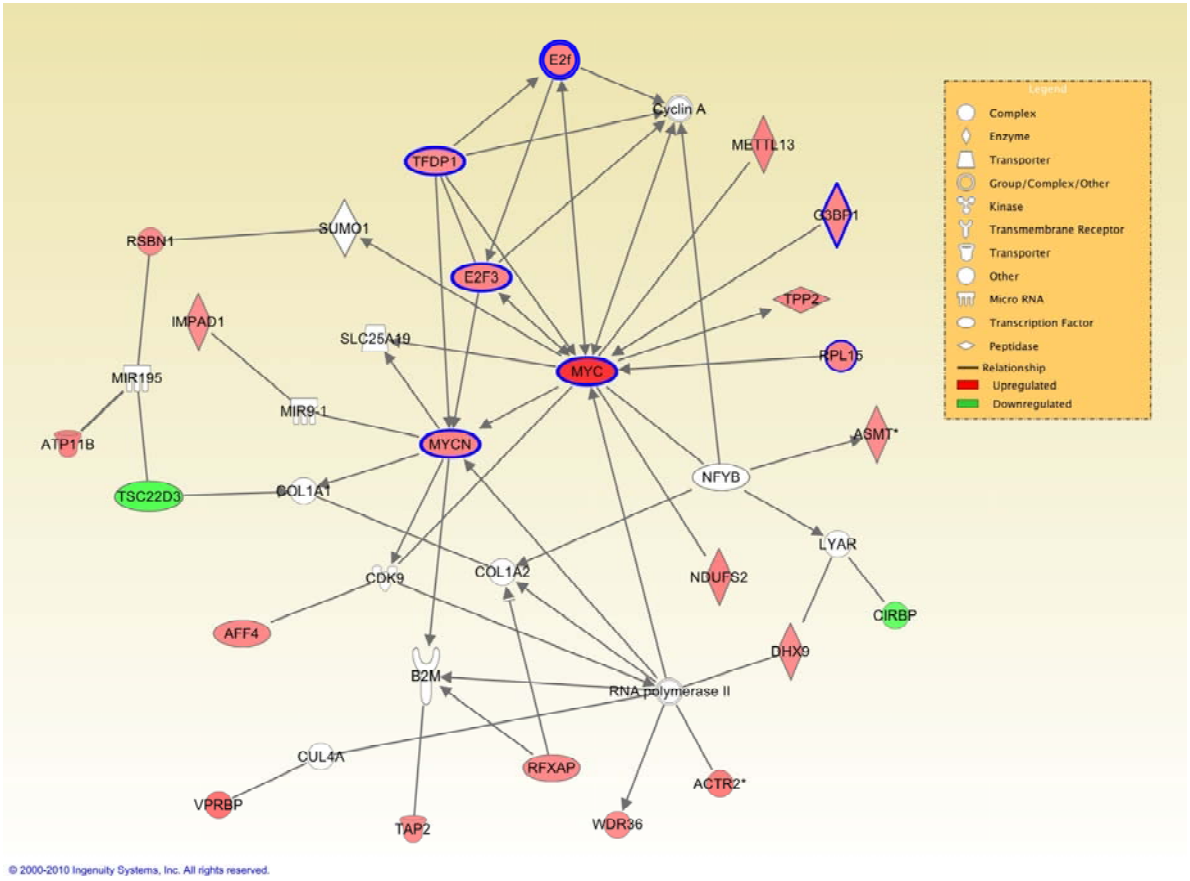


Figure 5.3: High-risk vs low-risk *MYC* in DLBCL (n=170) stratified according to BCL2 protein expression

Venn diagram representing the 18 cases with *MYC* translocations (left circle) and 19 cases with high *MYC* expression (right circle). 9 cases have both a *MYC* translocation and high *MYC* expression.
Low risk: *MYC* deregulation and BCL2 protein-negative
High risk: *MYC* deregulation and BCL2 protein-positive



Supplemental Figure 5.1: Pathway analysis of the *MYC* network

The darker intensity colors reflect more significant p values.

5.5 References

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Chapter 6: Conclusions

6.1 Summary

This work demonstrates that diminished CD20 protein expression as measured by FCM and *MYC* de-regulation, when associated with BCL2 protein expression, are associated with inferior survival in DLBCL patients treated with R-CHOP. Furthermore, the identification of high-risk patients can be detected at the time of diagnosis using technology that is currently available or compatible with most clinical laboratories and tertiary care centers (FISH). As such, both may become useful biomarkers that could complement the IPI once validated prospectively in an independent patient cohort.

Results from our CD20 work provide new insights in DLBCL biology

The observation that diminished CD20 expression is associated with poor survival in both R-CHOP and CHOP-only treated patients was unexpected and suggested that CD20 plays an important role in the pathology of DLBCL. This appears to be unrelated to the presence of mutations in the *MS4A1* gene, a finding which has been confirmed by others since the publication of our manuscript[1]. Walshe and colleagues have demonstrated that CD20 plays an important role in BCR signalling, which is known to be crucial for B cell proliferation and survival[2]. Others have shown that the binding of rituximab to CD20 directly inhibits BCR by preventing relocation to lipid rafts[3]. Chronic active BCR signalling is associated with the ABC subtype of DLBCL and in some cases may result from mutations in the BCR itself[4]. One hypothesis is that “dim CD20” may play a role in chronic active BCR signalling that could then lead to over-

expression of the NF-κB pathway. This would then lead to B cell proliferation, enhanced B cell survival and the inhibition of apoptosis through BCL2 over-expression (see Figure 6.1). Supporting this hypothesis was the observation that the "dim CD20" cases were predominantly BCL2 protein positive and of the ABC phenotype. Future work in this field is needed to fully elucidate the functional roles of both CD20 and CD19 in this disease.

Results from our CD20 work provide new rational targets for therapy

The therapeutic success of rituximab has stimulated efforts to develop improved anti-CD20 agents that are both fully humanized (2nd generation) to reduce immunogenicity and have an engineered Fc receptor (3rd generation) designed to improve therapeutic performance by adapting their effector functions (reviewed by Lim *et al*.)[5]. As highlighted in the introduction, there are at least three possible mechanisms by which anti-CD20 therapy enables B cell death; including complement dependent cytotoxicity (CDC), antibody dependent cytotoxicity (ADCC) and programmed cell death (PCD). While rituximab acts predominantly through CDC and ADCC (type I), some of the newer agents such as tosotusimab and GA-101 act predominantly through PCD (type II)[6].

There is recent evidence that suggests that the novel type II agents may be more beneficial in treating lymphomas with a "dim CD20" phenotype; a subtype we demonstrated was present in a subset of DLBCL, as described in chapter 3 of this thesis. Beers and colleagues have recently demonstrated that type II agents

are at least 5 times more potent at inducing B cell death than type I agents[7]. In addition to their enhanced capability of inducing direct PCD, type II agents are not internalized by the B cells and have improved ADCC because of a longer antibody half-life and exposure to surrounding macrophages. Indeed, cells such as the clonal cells in CLL, which have a "dim CD20" phenotype, rapidly internalize the CD20-rituximab complexes and degrade them in their lysosomes[7]. Thus, the level of expression of CD20 protein expression appears to be clinically important and may eventually affect the choice of anti-CD20 agent used in treating lymphoma patients. Further work into validating "dim CD20" as a biomarker in all B cell lymphomas is thus indicated.

Decreased CD20 expression after rituximab exposure may be more common than previously anticipated and may be due to down-regulation of CD20 mRNA at the pre and post transcriptional levels[8,9]. Dim CD20 expressing clones may be favoured in an environment of chronic exposure to anti-CD20 agents. Histone deacetylase inhibitors (HDAC) can increase mRNA expression of CD20 and sensitize cells to apoptosis after further exposure to rituximab however, they are less effective against lymphomas that over-express BCL2, which is often the case in DLBCL with "dim CD20"[9-11]. In contrast, prolonged exposure of rituximab-resistant cells to bortezemib, a proteosome inhibitor, can lead to a decrease in surface CD20 protein expression and a decrease in CDC after re-exposure to rituximab[12]. Thus modulation of CD20 expression prior to and following rituximab exposure is complex and likely clinically important. It appears

that some CD20-negative B cell lymphomas may still be re-sensitized to anti-CD20 agents when co-administered with HDAC inhibitors but not proteosome inhibitors[10,12].

Cooperation between oncogenes in DLBCL

The work provided in this thesis supports the classic model of cancer proposed by Hanahan and Weinberg almost 10 years ago[13]. Tumours evolve in a Darwinian fashion with a multi-step acquisition of genetic alterations that are crucial in the development and progression of cancer. They proposed that tumours acquire six different capabilities that provide them with survival advantages over their normal counterparts[13]. The biomarkers discussed in this thesis potentially impact at least four of these are capabilities (see Figure 6.2).

Insensitivity to anti-growth signals

Cell surface receptors such as BCR, possibly in conjunction with CD20, can transduce both growth and inhibitory signals to B cells. As such, cells acquiring mutations or phenotypes that would render them insensitive to the inhibitory signals of low affinity antigens would be favoured. It is possible that this "chronic active BCR" state may contribute to the global gene expression pattern of the ABC molecular subtype characteristic of lymphomas that are "frozen" in the "active" state and are unable to undergo terminal plasmacytic differentiation.

Self sufficiency in growth signals and limitless replicative potential

Over-expression of *MYC* would be advantageous on numerous fronts including self-sufficiency in growth signals and limitless replicative potential, especially when telomerase is constitutively expressed as in germinal center B cells. The information in Chapter 4 illustrates that *MYC* de-regulation can occur without clear evidence of a chromosomal translocation and in some cases, is presumed to be secondary to the activation of other oncogenic pathways. The detection of *MYC* translocations is currently possible in most tertiary clinical laboratories by FISH. Studying *MYC* expression as a biomarker would also be valuable as outlined in Chapter 5. Validating *MYC* expression as a prognostic marker using a nuclease protection assay or determining if MYC protein expression by IHC can be used as a surrogate for *MYC* mRNA expression is clearly indicated[14].

Inhibition of apoptosis

Inhibition of apoptosis is a hallmark of all cancers. Our work demonstrates that the inhibition of apoptosis through BCL2 over-expression, plays a major role in determining clinical response to R-CHOP in DLBCL cases that have concurrent *MYC* deregulation. The aggressive clinical behaviour of lymphomas harbouring concurrent *BCL2* and *MYC* translocations is well established. It is not unexpected that the addition of only one agent, rituximab, to CHOP would be insufficient to overcome treatment resistance in this disease. Recently, Beverly and Varmus have demonstrated that other anti-apoptotic members of the BCL2 protein family can also cooperate with *MYC* to induce and accelerate lymphomas in mice[15].

Although each anti-apoptotic protein appeared similar in maintaining cancer cell survival, there were dramatic differences in chemosensitivity that were due to the different half-lives of each anti-apoptotic protein. For example, the half-life of BCL2 is > 24 hours compared to < 1 hour for MCL-1. Chemotherapy given to MCL-1-dependent tumours (*MCL-1*/Eμ-*MYC*) resulted in apoptosis due to a proteosome-dependent degradation of existing MCL-1 levels and the prevention of new protein formation secondary to the effects of chemotherapy. In contrast, BCL2-dependent tumours (*BCL2*/Eμ-*MYC*) were chemoresistant due to persistently high levels of BCL2 protein in these tumour cells[16]. In human lymphoma cells, there are also different mechanisms that lead to the inhibition of the intrinsic apoptotic pathway that don't necessarily rely on the presence of BCL2 protein, however, like the mouse model, the levels of different BCL2 proteins themselves can be highly predictive of chemosensitivity *in vitro* [17,18].

It is interesting that lymphomas preferentially de-regulate BCL2 over other anti-apoptotic proteins given that they can also prolong cancer cell survival. BCL2 protein expression is present in all the indolent but incurable lymphoma subtypes and in ~ 60% of DLBCL [19]. The mechanisms by which BCL2 can be over-expressed differ between NHL subtypes and include *BCL2* translocations e.g. t(14;18)[20], *BCL2* gene amplification[21], promoter hypomethylation[22], transcriptional up-regulation of *BCL2* secondary to constitutive NF-κB activation and loss of micro-RNAs (mirs) 15 and 16[23]. In contrast, the other anti-apoptotic genes such as *MCL-1, BCL-XL and BCL-W* are seldom re-arranged or amplified in

comparison to *BCL2*. There is evidence that the *BCL2* gene, especially at the most common translocation breakpoint (MBR), has repeating DNA sequences that favours a "non-B" DNA conformation[24]. This non-classic DNA structure stabilizes single stranded DNA rendering it an amenable target for the RAG enzyme[24]. Other work suggests that the spatial allelic imbalance of *BCL2* on chromosome 18 is also a risk factor for rearrangements[25]. Given the longstanding history and high prevalence of *BCL2* de-regulation in lymphoma, BCL2 protein expression is routinely assessed by IHC in clinical laboratories as part of the diagnostic work up and thus can easily be incorporated as a biomarker in DLBCL without any additional costs or validation of the assay.

6.2 Current studies

A major collaborative effort is underway in British Columbia by the clinical lymphoma group and the Genome Sciences Center to discover novel genomic alterations in lymphoma using next generation sequencing technology. As a member of this team, I have participated in the accrual and processing of 92 DLBCL samples for whole transcriptome shotgun sequencing (WTSS). WTSS is a quantum leap forward in technological advancement in biological sciences, allowing the simultaneous sequencing of the entire "transcriptome" on one "array" platform[26]. This technology is more reproducible at detecting changes in gene expression than previous array based methods and outperforms the most recent and sensitive algorithm put forth by Choi and colleagues at classifying DLBCL molecular subtypes (98% concordance by WTSS in our series (unpublished)

versus 93% by IHC)[27]. It can also detect novel gene fusions and relative expression of mutant/wild type alleles.

Given that BCL2 protein expression appears to be very important in predicting outcome in the context of *MYC* translocations, we were surprised to discover that *BCL2* was by far the most mutated gene in our DLBCL transcriptomes; greater than BCL6 or *PAX5,* which are known targets of SHM, and significantly greater than any other gene in the extrinsic or intrinsic apoptotic pathways. Sanger sequencing of the *BCL2* gene (exons) in 340 DLBCL biopsies revealed 1041 novel variants in 249 samples and none in reactive tonsils. The ratio of transitions to transversions was very high ($R = 1.0$, $p < 10^{-16}$), where R should be 0.5 if there are no bias towards a specific mutation. These results are highly suggestive that these mutations are introduced by AID as a consequence of ongoing SHM in the germinal center.

BCL2 mutations have been described in the context of having a translocation t(14;18), where the translocated *BCL2* gene is targeted by SHM given its proximity to *IGH,* but the overall pattern and shear abundance of mutations suggest that they confer a favourable phenotype in DLBCL[28]. When mapping the location of the non-synonymous mutations against the functional domains of the BCL2 protein, 85% of the mutations clustered within the BH1 and BH3 domains and within the flexible loop domain, while sparing the domains that are important for anti-apoptotic functions (see Figure 6.3). The flexible loop domain has been

shown to play a role in delaying cells from entering the cell cycle (retards G1 →to S transition), allowing the cells that are exposed to various stresses additional time to repair DNA damage or remove intracellular reactive oxygen species (ROS)[29]. Furthermore, amino acids 32 to 68 appear to be critical in binding the P53 protein in response to DNA damage[30]. Thus BCL2 mutations may be an additional mechanism of synergistically enhancing the effect of *BCL2* by allowing cells to divide more rapidly and escape surveillance by P53, thereby further heightening the anti-apoptotic threshold. Considering that oral anti-BCL2 agents are currently being tested in clinical trials, it would be clinically relevant to investigate the role of *BCL2* mutations in lymphoma pathogenesis and response to therapy.

6.3 Future directions

Future work in biomarker research will likely rely heavily on the use of next generation sequencing technology. The cost of this technology is rapidly declining such that the “thousand dollar genome” should be within our reach in 2-3 years. Analysis of the 92 DLBCL transcriptomes is underway in British Columbia and is revealing an abundance of novel genomic alterations in a disease that is already genetically complex. The challenge will be to discover alterations that are both clinically and biologically important. This will require that novel discoveries are filtered against the survival data. Ideally all patients enrolled in clinical trials should provide lymphoma tissue at the time of study accrual, giving investigators the opportunity to make these necessary biological

and clinical correlations. This will only be realized by fostering meaningful collaboration between scientists and physicians, a step that might eventually bridge the gap between the bench and the bedside.

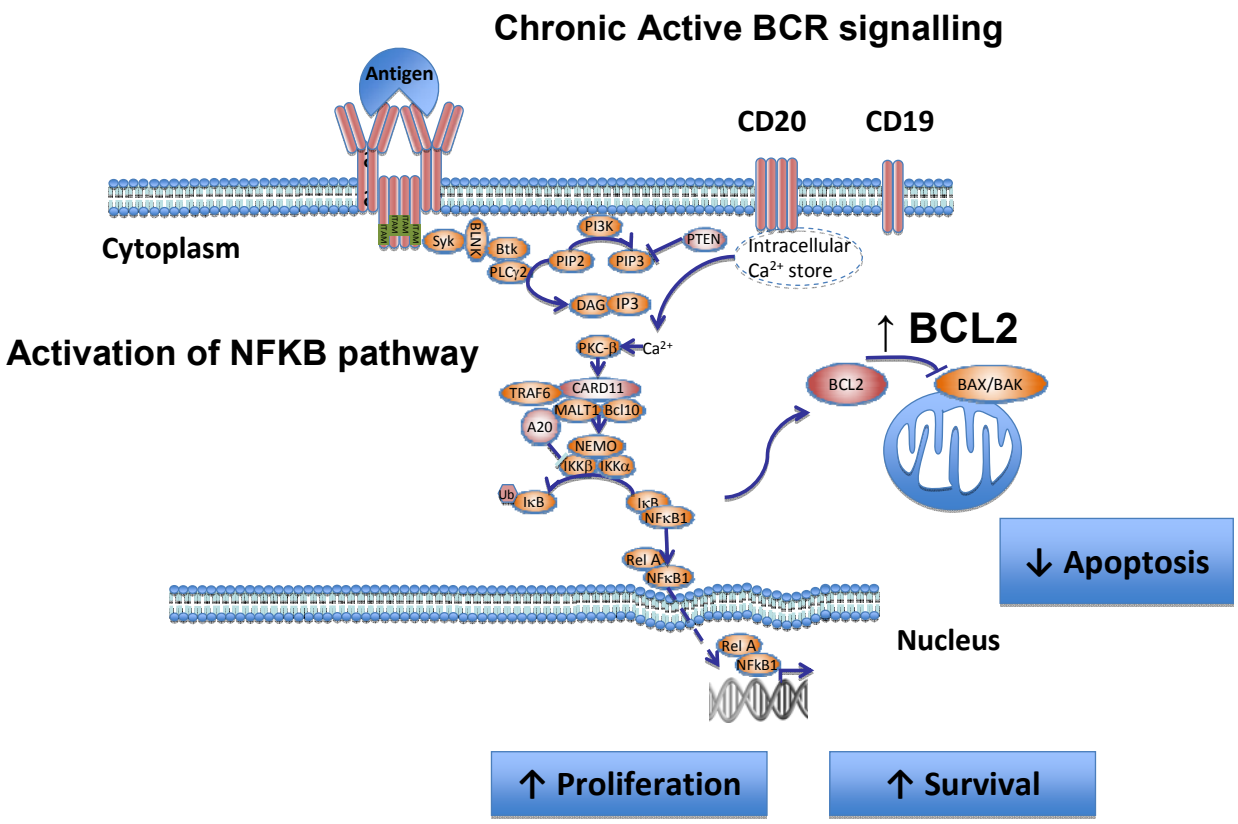

Figure 6.1: Pathways involved in chronic active BCR signalling

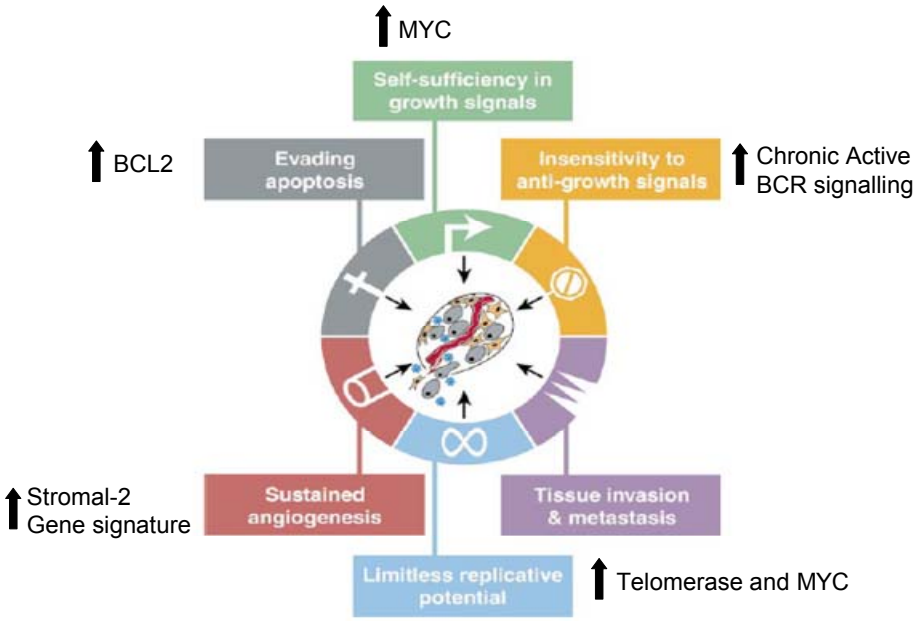

Figure 6.2: Adapted from Hanahan and Weinberg: The Hallmarks of Cancer (ref 13)

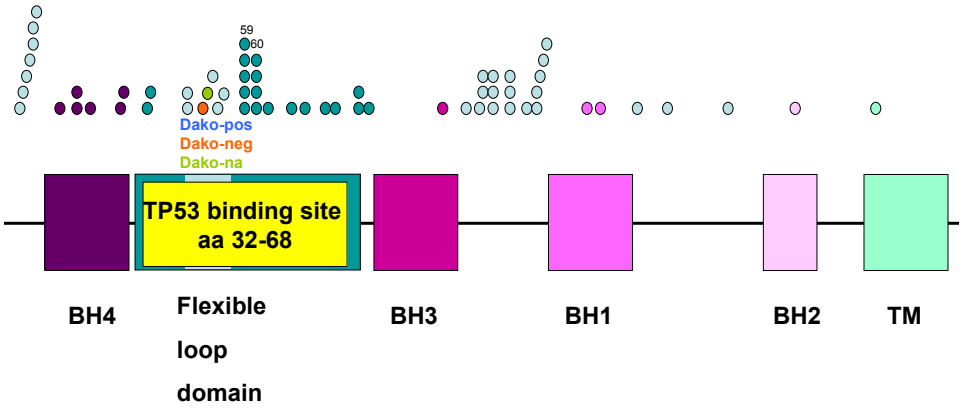

Figure 6.3: Incidence and location of mutations in the *BCL2* gene

Circles represent the presence of a mutation, the Yellow box represents the TP53 binding site

6.4 References

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Appendices

Research ethics board certificate of approval for Chapters 2 and 3

University of British Columbia - British Columbia Cancer Agency
Research Ethics Board (UBC BCCA REB)

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Certificate of Expedited Approval

PRINCIPAL INVESTIGATOR:	INSTITUTION / DEPARTMENT:	REB NUMBER:
Ryan Brinkman	BCCA/Terry Fox Lab (BCCA)	H08-00667

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
BC Cancer Agency	Vancouver BCCA

Other locations where the research will be conducted:
N/A

PRINCIPAL INVESTIGATOR FOR EACH ADDITIONAL PARTICIPATING BCCA CENTRE:

Vancouver:	N/A	**Vancouver Island:**	N/A
Fraser Valley:	N/A	**Southern Interior:**	N/A

SPONSORING AGENCIES AND COORDINATING GROUPS:

Michael Smith Foundation for Health Research

PROJECT TITLE:
Retrospective bioinformatic analysis of flow cytometry data of patients diagnosed with lymphoma

The UBC BCCA Research Ethics Board Chair, Vice-Chair or second Vice-Chair, has reviewed the above described research project, including associated documentation noted below, and finds the research project acceptable on ethical grounds for research involving human subjects and hereby grants approval.

EXPIRY DATE OF THIS APPROVAL: April 24, 2009

DATE DOCUMENT(S) APPROVED: April 24, 2008

CERTIFICATION:

1. The membership of the UBC BCCA REB complies with the membership requirements for research ethics boards defined in Division 5 of the Food and Drug Regulations of Canada.
2. The UBC BCCA REB carries out its functions in a manner fully consistent with Good Clinical Practices.
3. The UBC BCCA REB has reviewed and approved the research project named on this Certificate of Approval including any associated consent form and taken the action noted above. This research project is to be conducted by the provincial investigator named above. This review and the associated minutes of the UBC BCCA REB have been documented electronically and in writing.

UBC BCCA Ethics Board approval of the above has been verified by one of the following:

Research ethics board certificate of approval for Chapters 4 and 5

University of British Columbia - British Columbia Cancer Agency
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Certificate of Expedited Approval

PRINCIPAL INVESTIGATOR:	INSTITUTION / DEPARTMENT:	REB NUMBER:
Douglas E. Horsman	BCCA/BCCA/Pathology and Lab Medicine (BCCA)	H08-02834

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
BC Cancer Agency	Vancouver BCCA

Other locations where the research will be conducted:
N/A

PRINCIPAL INVESTIGATOR FOR EACH ADDITIONAL PARTICIPATING BCCA CENTRE:

Vancouver:	Douglas E. Horsman	**Vancouver Island:**	N/A
Fraser Valley:	N/A	**Southern Interior:**	N/A

SPONSORING AGENCIES AND COORDINATING GROUPS:
Canadian Institutes of Health Research (CIHR)

PROJECT TITLE:
Clinical impact of MYC translocation partners and Bcl-2 expression in High grade and intermediate grade lymphomas

The UBC BCCA Research Ethics Board Chair, Vice-Chair or second Vice-Chair, has reviewed the above described research project, including associated documentation noted below, and finds the research project acceptable on ethical grounds for research involving human subjects and hereby grants approval.

EXPIRY DATE OF THIS APPROVAL: April 23, 2010

CERTIFICATION:

1. The membership of the UBC BCCA REB complies with the membership requirements for research ethics boards defined in Division 5 of the Food and Drug Regulations of Canada.
2. The UBC BCCA REB carries out its functions in a manner fully consistent with Good Clinical Practices.
3. The UBC BCCA REB has reviewed and approved the research project named on this Certificate of Approval including any associated consent form and taken the action noted above. This research project is to be conducted by the provincial investigator named above. This review and the associated minutes of the UBC BCCA REB have been documented electronically and in writing.

UBC BCCA Ethics Board approval of the above has been verified by one of the following:

Dr. George Browman,	Dr. Joseph Connors,	Dr. Lynne Nakashima
Chair	First Vice-Chair	Second Vice-Chair

If you have any questions, please call:
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